Determination of Antiepileptic Drugs in Biological Matrices by LC/MS/MS with a Focus on Their Role in Forensic Cases

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By

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This thesis is dedicated to my wee man who enlightened my life with his giggling, honesty and impressed me by his patience and support. Many moments passed where I would have forgotten how tiny you were.
To my little angel

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Author’s Declaration

"I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution"

Signature _____

Printed name _____

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List of Abbreviations

AD	Anno Domini
ACN	Acetonitrile
AED	Antiepileptic drug
AEDs	Antiepileptic drugs
BC	Before Christ
CBZ	Carbamazepine
CBZO	Carbamazepine 10, 11 epoxide
CBZ-DiOH	10,11- Di hydroxy carbamazepine
CNS	Central nervous system
CI	Confidence interval
%CV	Coefficient of Variation percentage
R^2	Correlation Coefficient
CYP	Cytochrome isoenzymes
CSA	US Controlled Substances Act
DDDs	Defined Daily Doses
DFC	Drug Facilitated Crime
DMRM	Dynamic Multiple Reaction Monitoring
DUID	Driving under the influence of drugs
EI	Electron impact
ESI	Electro Spray Ionization
ESL	Eslicarbazepine Acetate
EU	European Union
FDA	Food and Drug Administration
FMS	Forensic Medicine and Science
FPHT	Fosphenytoin
GBP	Gabapentin
GABA	Gamma-Aminobutyric acid
GAD	Generalized anxiety disorder
GC	Gas chromatography
GC/MS/MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
NDTI	IMS Health National Disease and Therapeutic Index
Hrs	Hours

ISs	Internal Standards
Kg	Kilogram
L	Litre
LAC	Lacosamide
LTG	Lamotrigine
LC	Liquid chromatography
LC/MS	Liquid chromatography-mass spectrometry
LC/MS/MS	Liquid chromatography-tandem mass spectrometry
LEV	Levetiracetam
LIC	Licarbazepine
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LLOQ	Lower limit of quantification
LSD	Lysergic acid diethylamide
MeOH	Methanol
MDMA	Methylenedioxymethamphetamine
MHD	10-hydroxy carbazepine (or licarbazepine)
m/z	Mass to charge ratio
µg	Microgram
mg	Milligram
mL	Millilitre
MRM	Multiple Reaction Monitoring
NA-RTG	N-Acetyl retigabine
Neb	Nebulizing gas pressure
NHS	National Health Service
OXC	Oxcarbazepine
P.P	Protein precipitation
PBT	Phenobarbital
PGR	Pregabalin
PHT	Phenytoin
p-HPPH	p-hydroxy phenytoin
POM	Prescription only medicine
QC	Quality control
RTG	Retigabine

RUF	Rufinamide
R-LE	R-licarbazepine
S-LE	Eslicarbazepine
SIM	Selective ion monitoring
SPE	Solid Phase extraction
SPME	Solid phase micro-extraction
STP	Stiripentol
SUDEP	Sudden unexplained death in epilepsy
TDM	Therapeutic drug monitoring
TIC	Total ion chromatogram
TIG	Tiagabine
TLC	Thin Layer Chromatography
TLS	Traffic Light System
TPR	Topiramate
UGT	Uridine glucuronyl transferase
UPLC	Upper limit of quantification
UK	United Kingdom
USA	United States of America
v/v	Volume to volume
WHO	World Health Organisation
VIG	Vigabatrin
VPA	Valproic acid
Vd	Volume of distribution
ZNS	Zonisamide

Abstract

Antiepileptic drugs (AEDs) are prescription only medications which were firstly introduced in the 1880s to treat epilepsy. However, the rapid growth in the drug discovery market led to a new generation of AEDs with multiple mechanisms of action. These new drugs represent a promising treatment for many diseases in addition to epilepsy such as neurological disorders, psychological disorders and substance and alcohol abuse treatment as substitutes for benzodiazepines and methadone. However, their multiple roles triggered their misuse potential and concern on their abuse potential was raised in the literature, the media, and by many addiction organizations. Hence, this research highlights some of the AEDs which have raised concern and discusses their therapeutic effects, mechanism of action as well as their overdose and abuse probability from a forensic toxicology point of view.

Some AEDs have a narrow therapeutic index and require therapeutic drug monitoring in order to attain the optimum response. The majority of published analytical methods focuses on their analysis in serum and plasma within therapeutic ranges and includes a maximum of 11 AEDs in one analytical step. Therefore, a robust and accurate method was developed for the simultaneous analysis of 15 common AEDs and two of their major metabolites in whole blood using LC/MS/MS. The method was validated according to the standard practices for method validation in forensic toxicology (SWGTOX, May 2013) over a wide concentration range to include AED therapeutic and toxic concentrations which make it suitable for both clinical and forensic analysis.

Stability studies are of great importance in forensic cases where it takes up to a few weeks for autopsy, sampling, drug screening and finally confirmation analysis. However, reports specifically addressing the stability of antiepileptic drugs in whole blood are relatively scarce compared with those for drugs of abuse. Thus, using the previous method, the stability of AEDs in whole blood was investigated under different storage conditions.

The LC/MS/MS method developed for AEDs analysis in whole blood was successfully transferred to another laboratory and extended to include 18 AEDs and 4 metabolites. It was revalidated for AEDs analysis in serum and plasma in addition to whole blood. Before any new method can be adapted to routine forensic analysis, it has to be validated using authentic samples. A total of 467 previously processed samples were reanalysed using the

transferred method. The results were compared to the reference laboratory's values and these showed a very good correlation.

The prevalence of AED abuse, namely gabapentin and pregabalin, was investigated among prisoners. 904 urine samples were collected from 8 prisons in Scotland over a one month period. Firstly, a simple and accurate method was developed and qualitatively validated for 21 AEDs in urine to screen the urine samples. Secondly, the method was quantitatively validated for the positive AEDs.

Drug analysis in hair has multiple applications in clinical laboratories and forensic toxicology. However, only a few papers have considered conventional AEDs analysis in hair for therapeutic drug monitoring purposes. As part of this research, AED extraction from hair samples was investigated. Six different digestion methods and 4 clean-up procedures were compared for 16 AEDs. An LC/MS/MS method was qualitatively validated using the extraction procedure that attained the highest recovery with all AEDs. Subsequently, two authentic hair samples were tested and the method was quantitatively validated for the positive AEDs in these samples.

1 Antiepileptic Drugs

1.1 Introduction

In recent years, there has been a growth in reports of drug facilitated-crime following ingestion of drugs that depress the central nervous system (CNS) such as hypnotic and anti-anxiety drugs. In some reports, combinations of drugs or intoxicants containing legal as well as illegal substances have been reported to have been used. In other reports, unforeseen legal drugs have been found to have been misused. These cases make the list of drugs associated with forensic cases unlimited on the grounds that perpetrators may use any drug to help them to commit their offences either for self-harm or for harming others. Furthermore, any person working within the health sector should be aware of any drug with CNS effects (either depressant or stimulant) which is widely used and has the potential to be misused.

One group of legal medications which have increased awareness recently is the new generation of antiepileptic drugs (AEDs). This group has a wide range of effects on the central nervous system. Many of these show mood stabilizing effects, work as sedative and depressive agents and they are prescribed extensively for their effects on chronic neuropathic pain, anxiety and other psychotic disorders, although most of these indications are still unlicensed. Due to the rapid raise in their off-label indications, the prevalence of these drugs has significantly amplified during the last decade which increases their potential for abuse.

Debates in the press and scientific literature regarding these drugs and their probability to be misused were the reason they were chosen as a topic for this research.

1.2 History

Epilepsy is a neurological disorder that affects brain activity leading to abnormal firing of nerve cells, which manifests by seizures. Seizures are defined as a disturbance in the electrical activity of the brain that causes temporary changes in movement, awareness, feelings, behaviour, or other bodily functions. Their severity varies widely from one individual to another and depends on the type and size of the affected area of the brain (1, 2).

Epilepsy has been recognized since ancient times. The first known document to describe epilepsy, the Edwin Smith papyrus, goes back to ~3000 BC. Epilepsy at this time was considered a scary and unclean disease that was of demonic cause and its patients were treated by magic and religion. Magicians imposed a very strict diet in addition to the use of blood, bones and other human organs as a drink to cast out the devils. Religious treatment relied on sending the patients to sleep in temples until God appeared in their dreams and advised them about their cure (1, 2).

By the second century AD, the anatomy of the nervous system was beginning to be studied, and epilepsy was thought to arise either in the brain or from peripheral nerve stimulation. Therapies prescribed at this time were changing the diet and life style such as living in a warm dry climate, avoiding loud noises, sleeping and exercising regularly. More aggressive therapies included bleeding, skull cautery and trephination which was one of the more frequently used and widespread early surgeries (2). More simple regimens included herbals such as cardamom, plantain and tree fungi.

In the renaissance age, important modifications of the older theories were developed. It was believed that seizures resulted from an irritation of the brain and its membrane. This idea sets the stage for the advances in the modern era. Despite the rapid increase in understanding of physiological mechanisms of seizures in the 19^{th} century, primitive treatments were still in use like mistletoe, turpentine, trephining, castration and circumcision.

Bromides salts first appeared in 1857 and were the first successful pharmacological treatment for epilepsy. Potassium salts to treat hysterical epilepsy among young women were introduced by Charles Locock. Potassium bromide is a sedative compound that was used to counteract the excessive activity in the brain. However, it had toxic effects and diminished the mental function in addition to its sexual dysfunction effect. This successful treatment, besides its severe side effects, led to the introduction of another sedative drug, phenobarbital, in 1912, by Alfred Huaptmann (2, 3). In 2004, phenobarbital was one of the most widely prescribed AEDs because of its reasonable cost (4). Active investigation into other chemical compounds yielded hundreds of barbiturate derivatives, but most of these were toxic and not suitable for daily treatment.

In 1938, a new compound that shared similar properties to barbiturates but with fewer sedative effects, phenytoin, proved its high efficacy and came into use. Phenytoin is still

the most used AED in the United States (3). The next major drugs to be licensed were ethosuximide in 1958, carbamazepine licenced in the UK in 1965 and sodium valproate, first marketed as an AED in France. In the 1960s, benzodiazepines were introduced as an effective treatment for epilepsy specifically diazepam, lorazepam and clobazam.

Since the 1990s, there has been a noticeable increase in the number of antiepileptic drugs (AEDs) on the market. These include lamotrigine, topiramate, gabapentin, tiagabine, levetiracetam, eslicarbazepine acetate. Most of these have multiple mechanisms of action that facilitate their use in the treatment of a wide variety of CNS disorders (migraine, neuropathic pain, chronic pain conditions, psychiatric disorders, anxiety and panic disorders) in addition to their major indication as anti-seizure medication (5, 6).

Nowadays, epilepsy is considered the fourth most common disease after migraine, stroke and Alzheimer's disease. Epilepsy affects 50 million people worldwide, of which 80% live in the developing world (7, 8). In the United Kingdom, it was represented in 0.5-1% in 2002 of the adult population, increased to 0.95-1.11% in 2011 and the prevalence is still increasing (9, 10). The prevalence of new AED prescriptions has shown a 5-fold increase in children and adolescents in UK primary care between 1993 and 2005 even although the long-term safety surveillance data of these drugs is limited (11). New AED prescribing was highest among those aged between 20-50 years (12). A study of 21,551 nursing home residents found that 12% were taking an old generation AEDs such as valproic acid (13). In another study, 7.7% of nursing home residents were taking new AEDs with nearly half (42%) using the drugs for non-seizure conditions (14). The uptake of lamotrigine, topiramate and levetiracetam rose rapidly within the same period with domination for lamotrigine which accounted for 65% of all newer AED prescriptions (11).

Between 2001 and 2005, a comparison study on AED prescribing patterns was conducted in three different countries (United Kingdom, Netherlands and Italy). This research found a steady increase in the prevalence of all AEDs over these 5 years with a significant change in the United Kingdom but not in the Netherlands. Simultaneously, the prevalence of conventional AEDs decreased in the Netherlands and Italy, but not in the United Kingdom. The prevalence of newer AEDs showed a slight increase in all three countries. In 2005, lamotrigine use was highest in the Netherlands and the United Kingdom, whereas topiramate was dominating in Italy. The most significant increase was for vigabatrin which increased from 13% to 31% over this period whereas topiramate and levetiracetam had the highest number of prescriptions for younger people aged under 18 years (15). A

recently published study in 2014 assessed prevalence of AEDs over a nine year period (from 2001 to 2009) in five European countries; Denmark, Germany, Italy, Spain, the Netherlands and the United Kingdom. The study showed a steady linear increase in the use of AEDs between 6-15% each year. This increase was entirely attributed to the new generation of AEDs; particularly gabapentin, pregabalin, lamotrigine, levetiracetam and topiramate. The increase was majorly due to their off label use or being prescribed for indications other than epilepsy. On the other hand, the use of conventional AEDs was stable and did not show any significant change. The study also showed a slightly higher use of AEDs among female patients compared to male patients and their use increased with age (16).

1.3 AEDs Classification

AEDs are classified into new and conventional "old" AEDs depending when they first appeared on the market. All AEDs found and used before the 1990s are considered old AEDs. These include phenobarbital, phenytoin, ethosuximide, carbamazepine and sodium valproate whereas all AEDs appearing on the market after 1990 are considered new AEDs such as eslicarbazepine acetate, gabapentin, lamotrigine, lacosamide, levetiracetam, oxcarbazepine, pregabalin, retigabine, rufinamide, tiagabine, topiramate, vigabatrin and zonisamide (16).

1.4 AEDs and Their Potential Role in Forensic Cases

1.4.1 Off Label Use of AEDs in Non-Epileptic Medications

The term "off-label prescribing" refers to the use of a drug outside the terms of its marketing authorization or its product licence as known previously, including prescribing for unlicensed or unapproved indications. Generally, doctors have the right to prescribe a drug off-label but this will increase their professional responsibility (17). The off-label prescription of drugs affecting the central nervous system (CNS) such as mood stabilizers is well-known in the health care sector, especially by the psychiatric professions (18). However, the use of medication outside their approved indications may increase the risk of the side effects that could outweigh the potential benefits in addition to the ethical and legal issues related to the commercial promotion of the off label indications that have been raised recently (19-21).

In recent years, antiepileptic drugs have been associated with increased likelihood of off-label prescription in the non-epilepsy disorders even though most of these indications are still under investigation (22). The disorders in which AEDs have been noted as being effective include neurological disorders such as essential tremor, neuropathic pain and migraine and psychiatric disorders including anxiety, schizophrenia and bipolar disorders. This expected efficacy is mainly related to their mechanism of action which interferes with the disease process. For instance, topiramate and valproate are probably effective in treating migraine because of their ability to enhance GABAergic and reduce glutamatergic neurotransmission. Other drugs such as pregabalin, gabapentin, tiagabine, and levetiracetam are possibly effective in psychiatric disorders due to their enhancing effect of GABAergic transmission, whereas drugs that have blocking effects on voltage-gated sodium or calcium channels may be useful in treating neuropathic pain (such as gabapentin, pregabalin, carbamazepine, oxcarbazepine, lamotrigine and valproate). Another possible disorder where AEDs may be of clinical importance includes substance (23, 24) and alcohol abuse (25, 26).

In a study of data from the 2001 IMS Health National Disease and Therapeutic Index (NDTI) defining the prescribing patterns in the United States, Radley *et al* found an overall off-label use of 21% of which an estimated 15% lacked scientific evidence of therapeutic efficacy. Forty six per cent of these medications came from antiepileptic drugs. Gabapentin alone accounted for 83%. The problem was that only 17% of its prescriptions had scientific support compared with 66% without any support (27). Another study conducted in the St Andrew`s hospital, the largest psychiatric hospital in the United Kingdom, found that 28.5% of patients were treated with off-label mood stabilizers and 94% of these prescriptions were for unlicensed indications. The mood stabilizers most frequently prescribed off-label were: valproic acid (34%), carbamazepine (27%), lamotrigine (15.5%), topiramate (14%) and gabapentin (1.4%) while lithium, the licensed drug for psychiatric disorders, accounted for only 4% of all prescriptions owing to its side effects, toxicity, contraindications in other illnesses and compliance problems with blood monitoring (18).

Of all AEDs dispensed between 2004 and 2007, 71% was in epilepsy, 15% in psychiatry, 13% in neuropathic pain and <1% in migraine. Over these 4 years, the use of newer antiepileptic drugs increased from 40% to 49% in epilepsy, whereas it rose from 5% to 64% in psychiatry; mainly due to the increase in using lamotrigine, and increased from 79% to 95% in neuropathic pain as a result of the large increase in pregabalin prescriptions

(Figure 1-1). For migraines, the percentage increased from 72% to 96% due to topiramate and gabapentin while new AEDs use in trigeminal neuralgia increased from 3% to 16% where pregabalin and gabapentin dominated (28).

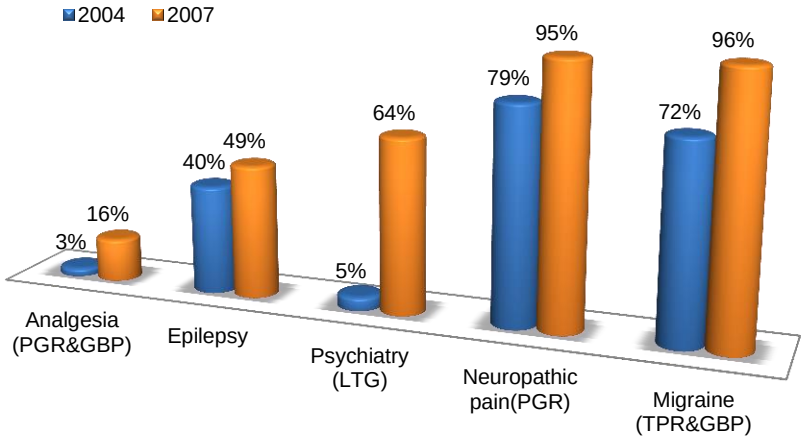


Figure 1-1: The Percentage of Increase in AEDs Use For Off-Label Indications Between 2004 and 2007.

The AED prescriptions have been increasing even though the proof for their indications is still unavailable. For instance, more than 83% of gabapentin sales came from off –label prescriptions (22, 27, 29). The main concern is that for most patients, gabapentin was not the optimal treatment (30). Off-label sales of zonisamide, topiramate, lamotrigine, and oxcarbazepine as well represented 66%, 53%, 52% and 50% respectively while tiagabine and levetiracetam had most of their prescriptions for authorised indications with 34% and 28% for off-label use (22). Compared with other practitioners, psychiatrists accounted for greater than 50% of the AED off-label expenditure (22). More than two thirds of them did not inform their patients about their off label medications because they considered that most of their patients did not have the mental capacity to understand the off-label concept and this information could adversely affect them (18).

A recent study published in 2012 showed that off label prescription in primary care was highest among CNS drugs including AEDs (66.6%) which represented the highest proportion followed by antipsychotics (43.8%) and antidepressants (33.4%). Specific AEDs with the highest off label use included gabapentin (99.2%). Indications that were most likely to be treated with off label AEDs were neurogenic pain (gabapentin and topiramate) and bipolar disorder (gabapentin and lamotrigine), chronic pain, fibromyalgia, and diabetic neuropathy (gabapentin) (19).

Table 1-1 summarises the most common off label indications of antiepileptic drugs and shows whether these indications are scientifically supported or not.

Table 1-1: AEDs Scientifically Supported and Non-Supported Off–Label Indications (5, 6, 25, 26, 31).

Drug	Anxiety	Insomnia	Essential tremor	Schizophrenia	Bipolar disorder	Neuropathic pain		Migraine	Substance abuse withdrawal
						Diabetic neuropathy	Trigeminal neuralgia		
Carbamazepine				○	●	○	●		○
Oxcarbazepine				○	●	○	●		
Gabapentin	● *	○	○			●		○	●
Pregabalin	●	○	●			●			●
Phenytoin						○	●		●
Lamotrigine			●		●	○	○		
Levetiracetam			○		○			○	
Tiagabine	○ *	○	○					○	
Topiramate	○			○	○			●	●
Valproic acid	○		●		●	○	○	●	●
Zonisamide			○					○	

* ●: Scientifically recommended, ○: No strong scientific support.

1.4.2 AEDs Accidental Overdose, Self-Poisoning Cases and Suicidality

The widespread use of the new generation of AEDs particularly in patients with psychiatric disorders, often as an unlicensed indication, increases the risk of self-poisoning (12). The frequency of AED intoxication in different studies was between 1.7% and 8% of drug related-poisoning (32). A study conducted between 2000 and 2007 in Edinburgh estimated the occurrence rate of AED overdose especially after increased used of these drugs in patients with psychiatric disorders who were at high risk of self-harm by drug overdose. There were 613 patients who ingested at least one AED. Carbamazepine, valproate, lamotrigine and phenytoin accounted for most of these cases (carbamazepine: 306, valproic acid: 163, phenytoin: 68 and lamotrigine: 63). The rest of the cases were 21 poisoning cases of gabapentin, 6 of topiramate, 6 of levetiracetam and 3 of pregabalin (12).

Another study was conducted over a period of 10 years (from 2002 to 2011) to evaluate the clinical effect of new AEDs in overdose. After excluding poly drug overdose and children under 15, they found that 347 cases met their inclusion criteria. The majority of these cases were gabapentin (116 cases), lamotrigine (67 cases), topiramate (56 cases), oxcarbazepine (55 cases), levetiracetam and tiagabine (15 cases each) and pregabalin (23 cases). Most of these cases resulted in mental status. Seizures were observed in cases of lamotrigine, oxcarbazepine and tiagabine. Lamotrigine was ranked the highest in terms of toxicity followed by topiramate, oxcarbazepine, gabapentin, pregabalin, tiagabine and levetiracetam was the lowest (33).

Many cases of individual self-poisoning by AEDs have been reported separately. Lamotrigine (34, 35), carbamazepine (36) and valproic acid (12, 37) poisoning reports were the most common in literature before the newer AEDs appeared. Topiramate comes after those drugs (38). Recently, poisoning cases of newer drugs seem to be reported more frequently. For instance, a deliberate overdose case of pregabalin (11.5 g) and lamotrigine (32 g) with a depressed level of consciousness was reported for a 29-year-old male in 2007. The plasma concentration of lamotrigine in this case was 45 mg/L. This concentration was the highest concentration ever recorded in the literature, while the plasma concentration of pregabalin was about 60 mg/L (39). Cases of suicide and intoxication by gabapentin have also been reported. Its effects varied from non-serious side effects (40) to coma (41) and death (42). The blood concentration in one of these cases was within the therapeutic range (42). In another suicidality case, gabapentin was ingested

with valproic acid and alcohol (37). Reports of acute tiagabine overdose have been published as well (43-45). In spite of their recent availability on the market and their wide range of safety, zonisamide (2005), lacosamide (2008) and levetiracetam (2014) have been reported in poisoning and attempted suicide cases (46-48), one of which ended in death (49).

As a result of these increased poison reports, a cross-sectional study was carried out to evaluate the risk factors associated with non-benzodiazepine AED intoxication. Deliberate self-poisoning accounted for the majority of AED intoxications. Different risk factors were found responsible for these cases such as psychological and physiological disorders and loneliness whereas higher education played a positive role in reducing the likelihood of self-poisoning (32).

Suicide is considered to be one of the most significant reasons for increased mortality among patients with epilepsy (5-7% of death cases) (50), bipolar disorder (one third of patients admitted to at least one suicide attempt) (51), and chronic pain conditions (52). Interestingly, 50% of currently available reports on suicide in epilepsy have been published in the last 10 years (53). Regarding their effect on mood, a meta-analysis study applied by the FDA in 2008 showed that suicidality occurred in 4.3 per 1000 patients treated with AEDs. As a result, they raised concerns that all prescriptions should be accompanied with a patient medication guide describing this risk on the grounds that all current AEDs pose an increased risk of suicidal thoughts and behaviour, regardless of their classification or mechanism of action (54). After the FDA alert, many researchers started to investigate suicidality in AEDs (50, 55-58).

A cohort study (2010) conducted by a team of researchers in Boston hospital over a five-year-period between 2001 and 2006 looked at prescription data for 15 AEDs used by 15 year-old patients and older. Among 297620 patients who used these drugs, there were 801 suicide attempts, 26 suicides and 41 violent deaths. They compared 13 out of the 15 drugs with two other AEDs (topiramate and carbamazepine). Topiramate is prescribed widely for different indications but not as a first line therapeutic approach whereas carbamazepine is usually prescribed for initial treatment of epilepsy. In this study, AEDs were compared with carbamazepine to investigate the risk of suicidal events in patients starting to use AEDs and they were compared with topiramate in cases of chronic AEDs treatment. As a result, five of these 13 drugs were found to increase the risk of suicide more than topiramate and carbamazepine (gabapentin, tiagabine, oxcarbazepine, lamotrigine and

valproic acid). Furthermore, this study found an increased menace in suicidal acts beginning within the first 14 days of treatment initiation, which means that AEDs may provoke behavioural effects prior to attaining their full therapeutic level (55).

However, few studies suggested that FDA concerns might be excessive and that the risk of not prescribing AEDs is far greater than their potential suicidal act (57, 59). They claimed that the major limitation of the FDA meta-analysis was the lack of a systematic data collection and that all these trials were not designed to evaluate the relation between AEDs and suicide, as a consequence, data gathering was not reliable. These studies suggested that the increased number of suicidality to be related to epilepsy itself. Available data is, however, not methodologically strong enough to support or reject the claimed increased risk of suicidality with AEDs. Hence, proper epidemiological studies investigating this phenomenon are prerequisites to understand whether AEDs are a precipitating factor in vulnerable individuals like epileptic patients. Moreover, clinicians need to pay attention not only to seizure patterns when choosing the appropriate AED but also to a number of different parameters, not least the mental state of the individual patient (59, 60).

1.4.3 AED Related Deaths

AEDs have been associated with many fatality reports and hepatotoxicity in younger people (<18) between 1964 and 2000. The new AEDs (vigabatrin, lamotrigine, topiramate, and gabapentin) were associated with 20 (30%) of 65 antiepileptic-treated death cases. Valproic acid had the highest percentage with 31 cases (48%) while carbamazepine, vigabatrin, lamotrigine and phenytoin were found in 11 (16%), 8 (12%), 7 (11%) and 3 cases (5%), respectively (61).

In Scotland, the number of AED related deaths increased from 1% in 2007 to 10% in 2012 resulting mainly from gabapentin cases (62). 44% of these cases were accidental poisoning, 14% mental disorder resulting from substance abuse, 16% intentional self-harm poisoning and 26% undetermined intent (Figure 1-2). Generally, death among epileptic patients results from epilepsy conditions including sudden unexplained death in epilepsy (SUDEP), status epilepticus, accidents and suicide.

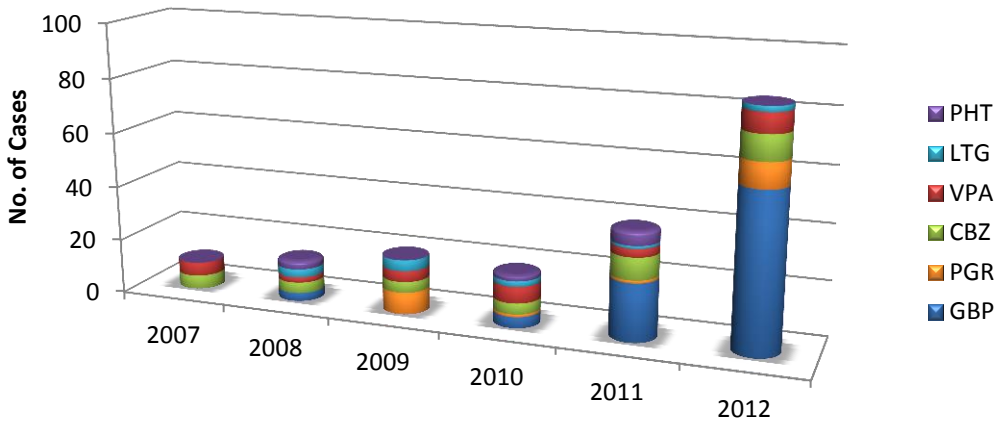


Figure 1-2: AED-Related Deaths in Scotland Between 2007 and 2012.

SUDEP accounts for approximately 17% of all epilepsy related deaths (63). It is defined as a sudden unexpected death in an epileptic patient in a reasonable state of health. The death occurs during normal activities without any obvious medical cause or evidence of seizures near the time of death (64). Different risk factors account for this phenomenon. The second most important factor after seizures frequency is the number of AEDs taken concomitantly. The risk is almost 10 times higher in patients taking more than two drugs compared to those who are on monotherapy (65, 66). Other factors contributing to SUDEP incidence are correlated to the variability of AED ingestion over time (67), alcohol abuse (68, 69) and to the usage of antipsychotic drugs (66). On the other hand, there are a number of studies which did not find any correlation between SUDEP and these factors (70).

1.4.4 AEDs and Online Pharmacies

As a consequence of both the ease and rapidity of access, the Internet offers a flood of drug-related data. The emergence of the internet as an unregulated source of controlled substances is an important development that may have significant public health implications (71). Advertising prescription drugs to patients directly is illegal in the United Kingdom and a high level of public awareness of this illegal trade of prescription drugs has been made. However, owing to the universal nature of the Internet, governments' bodies are incapable of controlling all of them. Moreover, most Internet drug vendors selling drugs without prescription usually do so to overseas customers to keep away from authorities in their countries (72, 73).

Psychonaut is a European Union, multi-site research project to investigate the developing relationship between the Internet and drugs with abuse potential. It involves 15 research

centres from eleven European countries. This project described the findings of the Scotland research site, which investigated the online availability of prescription drugs (74). An increased number of online pharmacies have been found to offer all kind of prescription drugs without prescription (74-76). Considering the issues of literacy, Internet access, and credit card ownership, it concluded that the increase access to Internet- sourced drugs may increase the role of the Internet in drug abuse in both higher and lower socioeconomic groups (74).

AEDs are available online in different sites. Examples of online pharmacies supplying AEDs online without prescription and forum sites of AEDs attended by AED misusers are shown in Table 1-2.

Table 1-2: Sites Attended by AED Misusers and Online Pharmacies Supplying AEDs.

Examples of Online Forum Sites Attended by AED Misusers (Websites Last Accessed 23 August 2015)
- **Erwoid, 2011**. Available from: https://www.erowid.org/experiences/exp.php?ID=85863. - **Stever, 2010.** Available from: http://aciddata.com/experiences/exp.php?ID=60394 - **Valhallen, 2003**. Available from: http://www.erowid.org/experiences/exp.php?ID=28984 - **BluelightForum (2005-2009).** Available from: http://www.bluelight.ru - **Opiophile (2009).** Lyrica recreational dosage/IV use. Available from: http://forum.opiophile.org - **Drugs-forum (2007-2009)**. Available from: http://www.drugs-forum.com/index.php
Examples of Online Pharmacies Supplying AEDs Inside and Outside the UK (Websites Last Accessed 23 August 2015):
- http://uk.popularpillsonline.net/. - http://auragenerics.com/index.php. - http://www.drugshoponline.com (pregabalin and gabapentin are advertised under the antidepressant drugs). - http://www.ipharmacylist.com. - http://www.eurodrugstore.eu. - http://www.77canadapharmacy.com/gabapentin.php. - http://www.omfgg.com/profiles/blogs/order-gabapentin-online-order. - http://firsthealthstore.net/products/neurontin.htm. - http://cheapest-shop24h.com/group.php?group_id=52 (levetiracetam is advertised as antianxiety and antidepressant).

Since the newer AEDs are not controlled drugs and the reports of their abuse uncommon, most of them are still available for sale online without any control over them, namely pregabalin, gabapentin and levetiracetam (77). Despite the lack of scientific reports regarding AED abuse in published literature, the situation is completely different on the World Wide Web (www), where information about drugs is just a click away. Tracking the www, it is clear that some AEDs, namely pregabalin and gabapentin, are increasingly misused by young people who are interested in using legal high and illicit drugs and sharing their experience with other users by posting on online forums. Another concern about online pharmacies is that some of these sites advertise for AEDs as antianxiety drugs without prescription.

1.4.5 AED Abuse Cases

Drug misuse is a deeply rooted social problem with serious consequences for public health and criminal fairness. Today drug selections are made from a variety of substances and, perhaps most importantly, the mix of drugs or intoxicants used contains legal as well as illegal substances (78). ”Problematic use refers to anyone who experiences social, psychological, physical or legal problems related to intoxication and/or regular consumption and/or dependence as a consequence of his/her own use of drugs or other chemical substances” as defined by The Scottish Consortium on Crime & Criminal Justice (78).

AEDs are a group of these legal prescription drugs which are found in abuse cases either alone or with alcohol (37, 79) and/or other common drugs of abuse in order to enhance their effects (37, 77, 79). These cases included carbamazepine, pregabalin, gabapentin, topiramate, phenytoin and lacosamide.

Unexpected abuse of few unscheduled sedating medications has raised concern over the liability of prescription drugs to be misused even though their preclinical abuse potential assessment studies implied that they would not be expected to produce any physical dependence of the CNS depressant type (80, 81). Programs for assessing the abuse potential of new chemical entities in humans were largely developed to be used in studies of the known drugs of abuse and a good correlation has been recognized between the signals detected in these methods and the actual abuse of substances in classes such as the opiates (82) and hypnotic drugs (83). A similar study has been conducted to investigate the reinforcing effects of levetiracetam (4000 mg), valproic acid (1500 mg), diazepam (30

mg) and diphenhydramine (400 mg). As a result, for the doses studied, the statistical findings of abuse potential were diazepam (9/10 measures significant) > levetiracetam (6/10) > diphenhydramine (5/10) > valproic acid (2/10) (81). Levetiracetam met the criteria for displaying abuse potential in this laboratory study even though no reports of actual abuse have been reported in the marketplace which make this finding difficult to elucidate (81). Maybe, the 4000 mg dose of levetiracetam used in the study is high enough (8-fold than the usual stated single dose) to consider the low liability of abuse. However, another explanation of these findings is that there is a significant risk of actual abuse of levetiracetam that has not been realized owing to its recent availability in the market (since 2000 in USA and Europe), the willingness of drug abusers to experiment with new drugs and as mentioned before, the ease with which information of drug effects can be shared on the Internet (81). Diphenhydramine is an example of an unexpected sedating drug. Pre-clinical studies carried out on diphenhydramine to investigate its liability of abuse did not recognize this effect and suggested that it had a low potential of abuse. The statistical findings of this study showed that the abuse potential of diphenhydramine is lower than that of levetiracetam, even though reports of actual abuse and fatalities from diphenhydramine have been recently reported (84, 85). The major drawback of such studies is that they do not assess the factors involved in the actual abuse of drugs (81), for example, the availability of other misused drugs, the cost or difficulty of getting them and the social circumstances of the abusers (86).

In the world of crime, lamotrigine, carbamazepine, phenytoin and valproic acid have been reported in drug-facilitated sexual assault cases (87-91). Actual abuse reports have been found in the press and scientific literature regarding a number of AEDs. Drugs mentioned are carbamazepine, valproic acid, phenytoin, gabapentin, pregabalin, topiramate and lacosamide.

1.4.5.1 Carbamazepine and Valproic Acid Misuse

The first report of AED misuse is for carbamazepine published in 1993 which describes two of several carbamazepine abuse cases. Both subjects were prescribed carbamazepine for alcohol withdrawal but they continued consuming the drug with alcohol after leaving hospital owing to their feeling of euphoria comparable to that of consuming alcohol with benzodiazepines (92). Another recreational misuse case report was in 1997 for a 20-year-old female who also abused carbamazepine with alcohol. She claimed that she had learned to abuse it “on the street” (93). Valproic acid has also been associated with alcohol abuse

(37). By the end of 1990s, both drugs had been mentioned in forensic literature as potential drugs of misuse (88, 90, 91).

1.4.5.2 Gabapentin Misuse

Gabapentin has been reported to be misused orally and intramuscularly in combination with other illicit substances such as: baclofen, cannabis, alcohol, SSRIs antidepressants (selective serotonin reuptake inhibitors), amphetamine, gamma-hydroxy butyric acid (GHB) (77) and lysergic acid diethylamide (LSD). People who misuse this drug say that their feelings are comparable to MDMA (methylenedioxymethamphetamine - known as ecstasy), amphetamine, fully–sedated opiate, dextromethorphan, and cannabis abuse. It causes euphoria but its tolerance level is very high (77). The first reported case of gabapentin abuse in the literature appeared in 1997 (94). This case involved a 41-year-old woman, who had been consuming crack cocaine for at least twelve months. During her rehabilitation from cocaine abuse, she had started using her husband`s prescribed gabapentin voluntarily to substitute her cocaine dependence. She said that it was useful in diminishing her cravings, but after three months she had resorted to drug-seeking behavior. Another case was a 67-year-old women who had a history of alcohol abuse leading to polyneuritis; a simultaneous impairment of function of many peripheral nerves such as the cranial nerves (95). She was prescribed gabapentin as a pain relief, but she developed tolerance toward the drug which resulted in doubling her dose to 4800 mg/day, but she actually increased the dose to 7200 mg/day by herself to get an acceptable analgesic effects. When she ran out of her drug, withdrawal symptoms appeared and she was hospitalized.

Secondary to the wide spread use of gabapentin in correctional facilities, reports of gabapentin abuse have been published describing inmates who snort gabapentin powder from capsules during their incarceration (96, 97). Most of them were cocaine abusers who intended to misuse gabapentin as a way to get high, feeling similar to that following cocaine use. These concerns about the potential abuse of gabapentin started to arise in 2001 when the medical staff at several correctional institutions in Florida noticed that inmates were requesting refills of gabapentin capsules sooner than scheduled and often making allegations that their medication had been stolen by others. They were prescribed gabapentin for assorted medical and psychiatric conditions (neuropathic pain, epilepsy, anxiety and mood disorders) and they were allowed to keep their prescription bottles themselves. After investigation, the medical staff discovered that only 19 out of 96

prescription bottles were found to be in the possession of the appropriate patients (96). As a result of these findings, gabapentin was removed from the department of correction formulary in August 2001, because it was noted that gabapentin was not abused for its sedating properties but for its effects of getting high, especially after intranasal ingestion, as admitted by the inmates themselves (96).

Individual cases of gabapentin abuse have been increasingly reported in different places (98-100). In some cases, it has been abused with heroin (100), alcohol (98) and other substances of abuse (77). In most cases reported, abuse of gabapentin occurred during the recovery program of alcohol abuse (95) or cocaine abuse (94). Furthermore, there are cases in which gabapentin abuse was by epileptic patients themselves who described having euphoric feelings after using it as a treatment (99). More details about these cases can be found in Appendix 1-1. In all these cases euphoria and "substance abuse" like feelings were reported. Symptoms of withdrawal syndrome were associated with gabapentin discontinuation, (confusion, agitation, nervousness, anxious, headache), even when it was used within the therapeutic dose (1600-4800 mg/day)(101). This syndrome was found to be similar to that of benzodiazepines and alcohol (102).

1.4.5.3 Pregabalin Misuse

Pregabalin use has rapidly increased from 4.6 million Defined Daily Doses (DDDs) in 2007 to 9.3 million in 2009 (103). A controlled clinical study carried out on over 5500 patients showed that self-reporting rates of euphoria ranged between 1-12% (104) and the effects seemed to be dose-related (77). These effects are summarized in Table 1-3.

Table 1-3: Pregabalin Effects–Related Dose as Described by its Misusers (77).

Dose	Reported Symptoms
600 mg	Disorientation, increased physical and psychological awareness, difficulty to drive, slurred speech, hallucinations.
900 mg	Strong feelings of drunkenness, difficulty to walk, little euphoria.
1200 mg	Drowsiness, euphoria.
>1500 mg	Uncontrolled drowsiness, frequent hallucinations, great euphoria, Dextromethorphan-like dissociative effects.

Another study reported 16 cases of pregabalin abuse between 2007 and 2009 (103). Most of these were for patients with a median age of 29 years who were prescribed pregabalin for treatment of generalized anxiety disorder (GAD). Six out of these 16 cases were intentional abuse with maximum daily doses ranging from 300 to 4200 mg, mostly taken as a single dose. As a result, this study suggested that pregabalin is likely to be associated with an abuse potential (103).

Pregabalin was reported to be misused in combination with alcohol, prescription drugs (zopiclone, benzodiazepines, and gabapentin), Illicit/recreational drugs (marijuana, heroin/opiates, amphetamines and LSD) and with the "Legal High" drugs such as mephedrone and salvia divinorum. Its tablets have been taken by different routes: orally (parachuting), intravenous after dissolving the tablets in water, rectal (plugging) and by inhalation. Users state that dose for dose, pregabalin outshines gabapentin, however tolerance is gained more quickly (77, 103).

Pregabalin withdrawal symptoms have been also reported in many cases. One of them was for a 47-year-old man who was consuming pregabalin with alcohol and cannabis at irregular intervals. He developed vegetative withdrawal syndrome when he attempted to wean himself off pregabalin (105).

Since 2012, several articles have been published questioning the potential of gabapentin and pregabalin; which are known as "gabapentinoids", to be abused (24, 106-108). A questionnaire-based survey was carried out in six substance misuse clinics in Edinburgh. The results showed that 22% of respondents admitted to abusing gabapentinoids, and of these, 38% abused them in order to potentiate methadone-like effects (109). Most gabapentinoid abusers were individuals with a history of recreational poly-drug misuse, who self-administered these drugs with excessive dosages up to 3–20 times of clinical dose. Hence, it has been advised that physicians considering prescribing gabapentinoids for neurological/psychiatric disorders should carefully evaluate for a possible previous history of drug abuse (108).

1.4.5.4 Phenytoin Abuse

Reports of phenytoin misused with marijuana and cocaine by inhalation have been reported since 1993 (79, 110, 111) . In two of these reports, phenytoin was abused with crack cocaine. Abusers tended to mix phenytoin with crack before the drug was smoked (110). Recently, a case was found of a 19-year-old man who combined phenytoin, marijuana and alcohol recreationally based on a rap song (79). Phenytoin has also been detected in sexual assault cases (88).

1.4.5.5 Topiramate Abuse

Topiramate has also been misused by a bipolar patient with an eating disorder in order to lose more weight. She refused to use valproic acid and lithium because of their side effect of weight gain. Due to this decision, she was prescribed topiramate. Two weeks later, she had decreased cognition, dulled thinking, moderate sleepiness, and a gastrointestinal disorder. After her examination, it was obvious that she had increased her dose voluntarily when she noticed that she started to lose weight. This positive advantage of topiramate over other anti-manic agents, may result in its abuse by those patients with obesity body-image disorder (112). Another case was reported of topiramate abuse by a 17-year-old female. She ingested 800 mg topiramate intentionally in order to get high. She fell down nonresponsive and became combative and confused with significant speech impairment (113).

1.4.5.6 Lacosamide Abuse

No actual reports have been found for lacosamide abuse, but clinical studies conducted on this drug indicate that it has the potential for abuse, especially by recreational abusers who described its effect as similar to alprazolam. It produces a kind of sedation with high euphoric drunk and drug like effects. Healthy subjects in these studies reported a high rate of euphoria-type response but no liability to produce physical dependence has been recorded (114).

An overview of relevant data from the reports mentioned above can be found in Appendix 1-1.

1.4.1 AEDs, Alcohol and Substance Abuse Treatment

Carbamazepine, phenytoin and valproic acid are AEDs used in the treatment of alcohol withdrawal (26). As a result of perceived low abuse potential, many studies have investigated the role of AEDs in the rehabilitation of substance (23, 115-117) and alcohol abuse as alternatives to benzodiazepines (25, 31). However, reports of AED abuse with alcohol and other substances of abuse have recently increased and awareness of this problematic issue has been raised in the press and scientific literature (24, 37, 77, 79, 103, 106-108). This indicates that the potential for abuse may have been underestimated.

1.4.2 AEDs and Driving Cases

Driving under the influence of drugs (DUID) whether prescribed medication or illegal substances is an issue of growing concern in industrialized countries, as it is a risk factor for and a cause of road accidents and is considered as dangerous as driving under the influence of alcohol (118). Drugs may lead, alone or in combination with alcohol or other CNS influencing compounds, to slower reaction times, sleepiness, poor concentration, distorted perception, overestimation of one's abilities (leading to unnecessary risk-taking), aggression until panic attacks, or blurred vision due to widened pupils. As a consequence, the number of accidents attributed to the consumption of psychoactive substances is still increasing. For these reasons, many countries consider driving whilst controlled substances are present in the blood to be an executive offence and, in the case of additional signs of impairment or an accident caused by any intoxicant, a criminal offence (119). In the UK, it is an offence under the Road Traffic Act of 1988 to drive under the influence of drugs or medicine and in most cases this offence would be considered in the same way as a drink driving conviction and carry similar penalties (120).

AEDs are considered to have the potential to impair a driver and should be tested in cases of suspected impaired driving (121). A study conducted between 2003 and 2007 in the USA to evaluate the prevalence of gabapentin in impaired driving cases found 137 positive gabapentin cases. Seven per cent of these accident cases were positive for gabapentin alone with the remaining 93% showing multiple drug use (122).

Nowadays, scientists are calling for a new way of highlighting the dangers of driving after using drugs. They want a "traffic light" system, with red, amber or green markings indicating whether it is safe to drive after taking a medicine or not. The red mark means

that the drug should not be used by drivers, the amber one means that the driver should take advice, whereas the green mark means that the drug should not affect driving performance. They claimed that there is a widespread confusion among the population about what is safe for driving and what is not (123). This system is still under investigation and has not been applied yet in most countries.

1.5 AEDs and Legislations

AEDs are not controlled by either the Misuse of Drugs Act 1971 of the UK, the Misuse of Drugs Registrations 2001, or the Poison Act 1972 (124, 125). AEDs are classified as prescription only medicines (POM) under the Medicine Act 1968 (126). Recently, pregabalin has been added to the list of new recreational psychoactive substances and officially notified to the relevant EU agencies (127). With regards to the US Controlled Substances Act (CSA), pregabalin and lacosamide are classified in schedule V (114, 128).

Recently, new guidance on prescribing responsibilities for selected medications has been applied. It aims to provide a clear understanding of where clinical and prescribing responsibility rests between specialists and GPs. It is called The “Traffic Light System (TLS)”. This system divides drugs in categories depending on the patient safety, ensuring appropriate usage and the presence of a shared care protocol. These categories are red, amber, green and grey. The red group includes drugs majorly prescribed by consultant or other physician within a secondary care service, drugs with new prescribed indication, new drugs in the markets and drugs not listed in the NHS references. The amber group includes drugs that are usually initiated by a specialist but for which GPs may agree to continue ongoing prescribing, drugs that have shared protocols or treatment guidelines. The green group includes drugs for which GPs take full responsibility for initiating and ongoing prescribing whereas the grey category includes drugs that lack evidence of clinical effectiveness, cost prioritization or drugs that have concerns over their safety. With regards to the Traffic Light Drugs List published in 28 June 2011, AEDs are classified under the amber category except gabapentin and pregabalin which are classified under the green category in case these drugs are prescribed for neuropathic pain (129).

1.6 AED Chemistry and Pharmacology

1.6.1 AED Chemical Structures

AEDs are acidic, basic and amphoteric compounds with a wide range of pKa and different polarities as indicated in Figure 1-3.

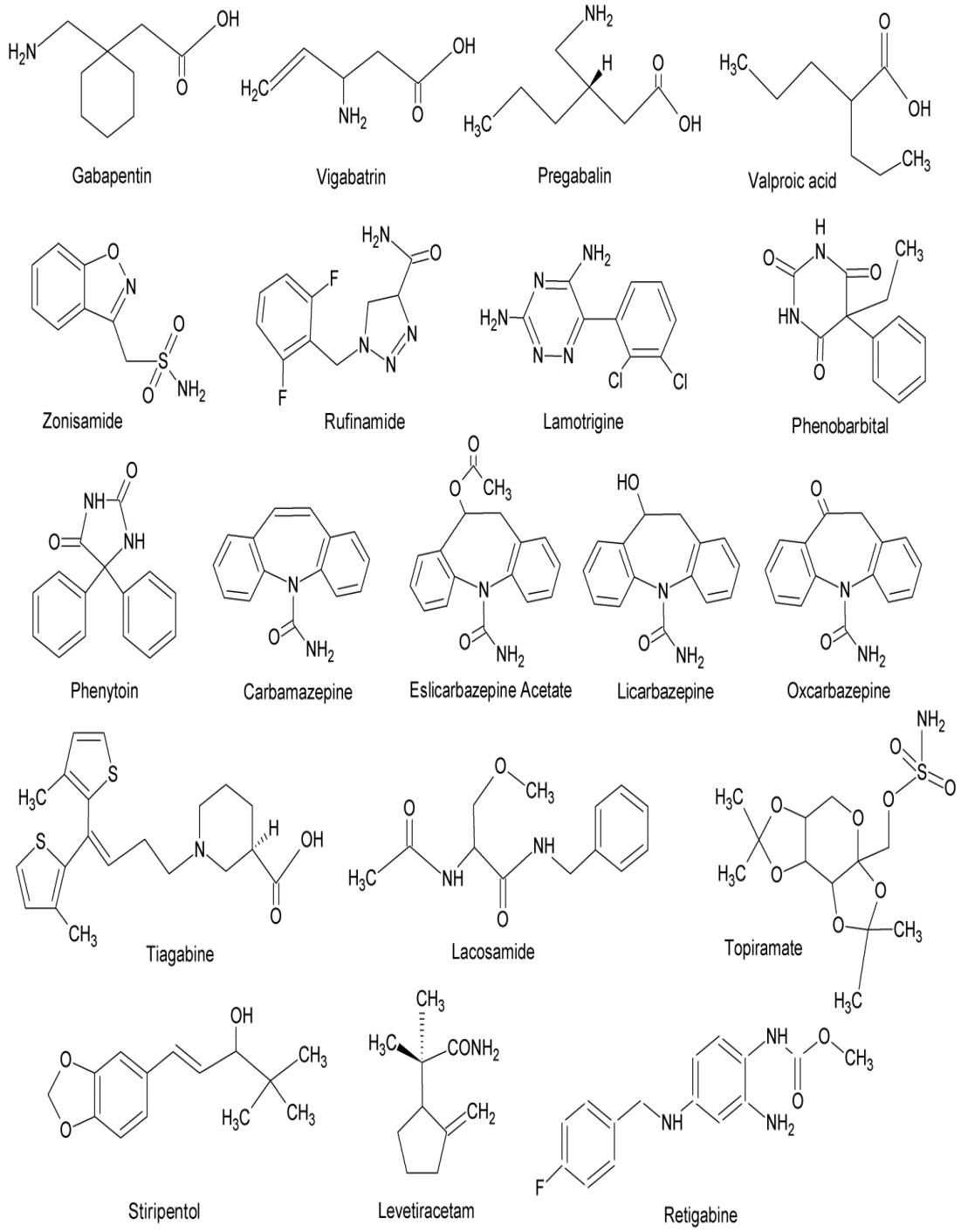


Figure 1-3: Chemical Structures of 18 Commonly Used AEDs and 4 of Their Main Metabolites.

Old AEDs represent the core structures for the development of subsequent second and third generation of new AEDs. For instance,

a. Phenobarbital was the first successful AED derivative of barbituric acid and it was used subsequently in primidone and phenytoin synthesis. These are second generation AEDs. Fosphenytoin is a derivative of phenytoin and therefore a third generation AED to phenobarbital.

b. Carbamazepine is an iminostilbene chemically related to the tricyclic antidepressant; imipramine. Its structure is the base for oxcarbazepine, a second generation AED which in turn represents the core for eslicarbazepine acetate synthesis, a third generation AED to carbamazepine.

c. Valproic acid is an achiral short branched fatty acid with 8 carbons without any nitrogen atom or cyclic ring and represents the core for a few derivatives such as valpromide and valnoctamide.

AEDs produced after 1990s have a wide variety of new chemical structures and are not considered as a second generation to existing AEDs. Some new AEDs contain the GABA moiety in their structure such as vigabatrin, tiagabine, gabapentin and its second generation pregabalin. Lacosamide is an N-benzyl-N-acetamide derivative of serine. Lamotrigine is a triazine and rufinamide is a triazole but they are not chemically similar. Levetiracetam is a heterocyclic amide related to piracetam which is a cognitive enhancer. Retigabine is a carbamic acid ethyl ester derivative. Topiramate is a sugar derivative from D-fructose and acetone and finally zonisamide is a benzisoxazole derivative (130, 131).

1.6.2 AED Pharmacokinetics

A summary of AED pharmacokinetics are presented in Table 1-4 (130, 132-137).

Table 1-4: A Summary of AED Pharmacokinetics.

Drug	Half Life (Hrs)		Blood concentration			Vd (L/kg)	Blood/ plasma	Protein Binding (%)
	Chronic therapy	Single dose	Chronic therapy (mg/L)	Single dose (mg/L)	Time to Peak Concentration (Hrs)			
Carbamazepine	18-65	5-26	1.7-15	4-8	3.2-5.7	0.8-1.8	0.6	75
Eslicarbazepine acetate	10-16	13-21	8.8-25	7.8-16	2.1-3.3	0.7	1.2-1.4	40
Lacosamide	13	-	2.5-13.5	-	1-5	0.5-4	n/a	15
Lamotrigine	12-62	29	2.3-5.6	0.4-1.6	1.9-2.1	1.9-1.3	n/a	55
Levetiracetam	6-10	7.8	29-31	14-51	1-4	0.4-0.7	0.8-0.9	<10
Oxcarbazepine	1-3	-	0.05-1.2	1	1.3	3-15	0.5	40
Gabapentin	5-9	-	1.9-2.6	2.2-6.1	4-8	0.8-1.3	1.0	<3
Phenobarbital	48-144	-	10-48	0.7-18	1.9	0.5-0.6	0.8-0.9	50
Phenytoin	8-60	17	7.8-20	1.6-14	2-5.2	0.5-0.8	0.5-0.6	87-93
Pregabalin	5-11	9.1	1.3-4.9	1.9	1	0.5-0.6	n/a	0
Rufinamide	5-11	8.8	2.6-4.7	2.2-4.3	5.4	0.8-1.2	1	26-35
Retigabine	7-11	7.4-9.2	0.49-0.98	0.41-0.11	1.4-1.8	2-3	0.6-0.7	60-80
Stiripentol	4.5-13	-	4-22	-	1.5	1.03	n/a	99
Tiagabine	4-9	-	0.03-0.16	0.06-0.13	1-2.2	0.8-2.1	n/a	96
Topiramate	19-23	-	2.4-27	1.7-7.7	1.4-3.5	0.5-0.8	1.3-7.1	15
Valproic acid	8-12	9	50-100	32-90	1.5-3	0.1-0.4	0.5-0.6	90-95
Vigabatrin	6-8	-	75	18-77	0.8-0.9	0.8	0.6-0.9	0
Zonisamide	53-75	63	21-28	2.9-6.4	2-5.3	0.7-1.8	4-5	40

1.6.3 Metabolism and Excretion

1.6.3.1 Carbamazepine

Carbamazepine is a type of tricyclic antidepressant. It is extensively metabolized to 10, 11-dihydroxycarbamazepine via 10, 11-epoxide formation with subsequent hydrolysis with only 1% excreted unchanged in urine (Figure 1-4). Another minor pathway results in iminostilbene formation. Its three major metabolites are 10,11-dihydroxycarbamazepine (10-20%), carbamazepine-10-11-epoxide (2%) which has an anticonvulsant activity similar to that of the parent drug and iminostilbene (0.5%) (137).

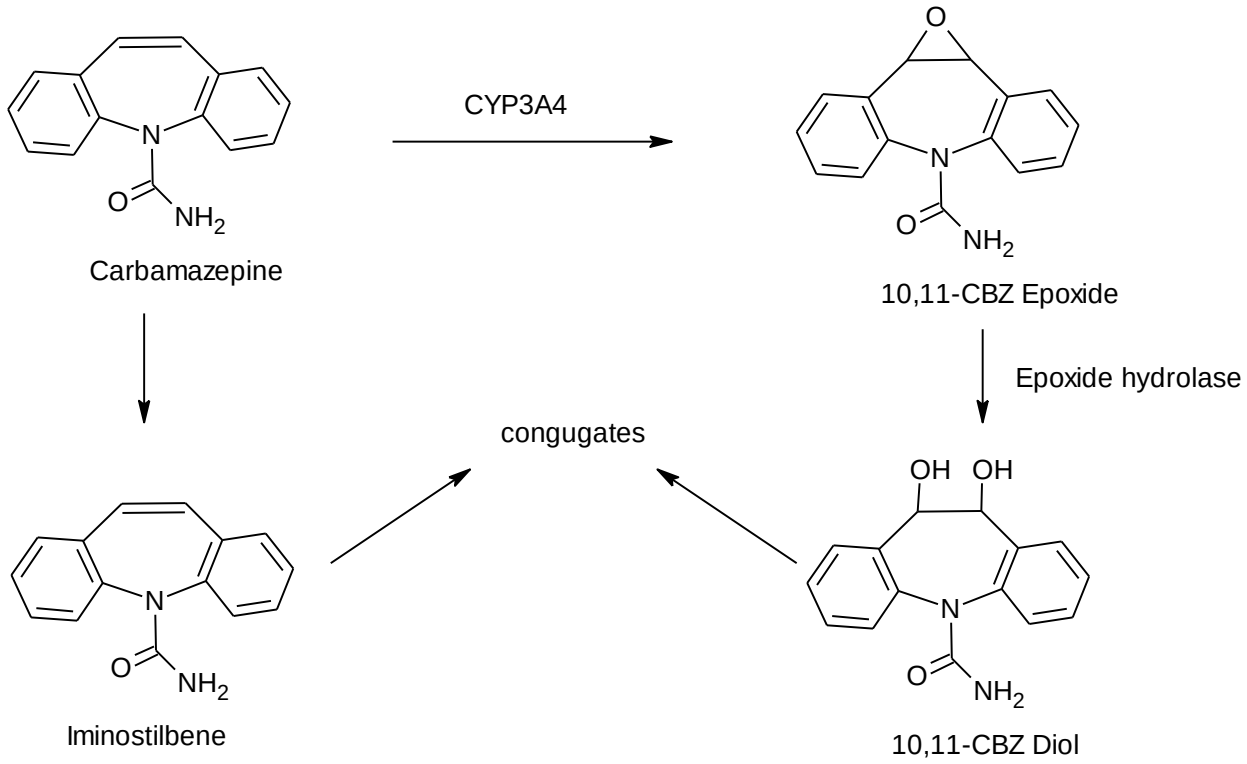


Figure 1-4: Carbamazepine Metabolic Pathway.

1.6.3.2 Eslicarbazepine Acetate and Oxcarbazepine

Eslicarbazepine acetate is a second generation AED to oxcarbazepine and third generation to carbamazepine. It is a prodrug of S-licarbazepine which is a mono hydroxyl metabolite (S-MHD) of oxcarbazepine and the main active metabolite responsible for the antiepileptic effect of both oxcarbazepine and eslicarbazepine acetate. As the MHD could not be patented, the favourable pharmacokinetic profile of S-licarbazepine was one of the incentives for the development of ESL as a new AED. Eslicarbazepine acetate is rapidly hydrolysed to S-licarbazepine only which in turn oxidises to oxcarbazepine and reduces to trans-diol carbazepine, whereas oxcarbazepine as a drug is metabolized to both S-

licarbazepine (80%) and R-licarbazepine (20%) (Figure 1-5). All their metabolites are subject to conjugation with glucuronic acid prior to their urinary excretion; conjugated S- and R-licarbazepine and trans-diol carbazepine (137, 138).

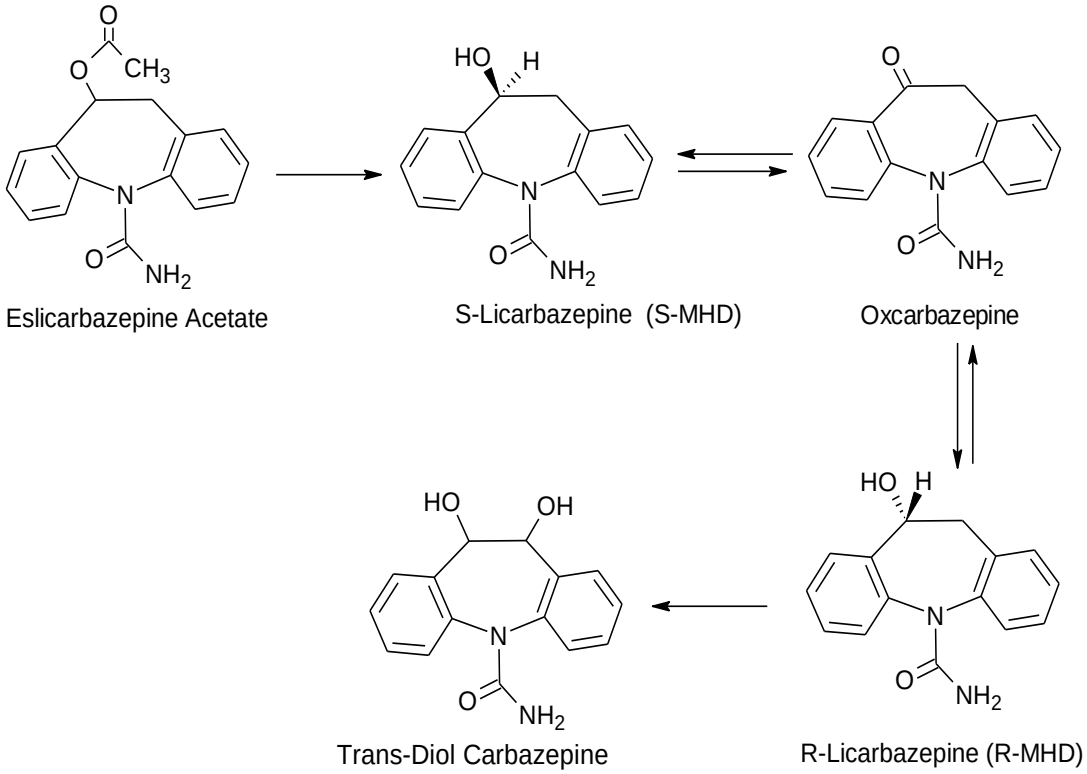


Figure 1-5: Eslicarbazepine Acetate and Oxcarbazepine Metabolic Pathway.

1.6.3.3 Gabapentin

Gabapentin is a gamma amino butyric acid (GABA) analogue. It is not metabolized, not bonded to plasma protein and is eliminated unchanged by the kidneys (76-81%) and by faeces (10-32%) (137).

1.6.3.4 Lacosamide

Lacosamide (SPM 927, Harkoseride: as known previously) is a functionalized amino acid molecule. Its urinary excretion profile shows 40% as unchanged parent drug, 30% corresponding to its main metabolite desmethyl lacosamide and 17% to unknown polar fractions (Figure 1-6). Minor fractions were biotransformed to hydroxyl lacosamide and descarbonyl lacosamide (132, 139) .

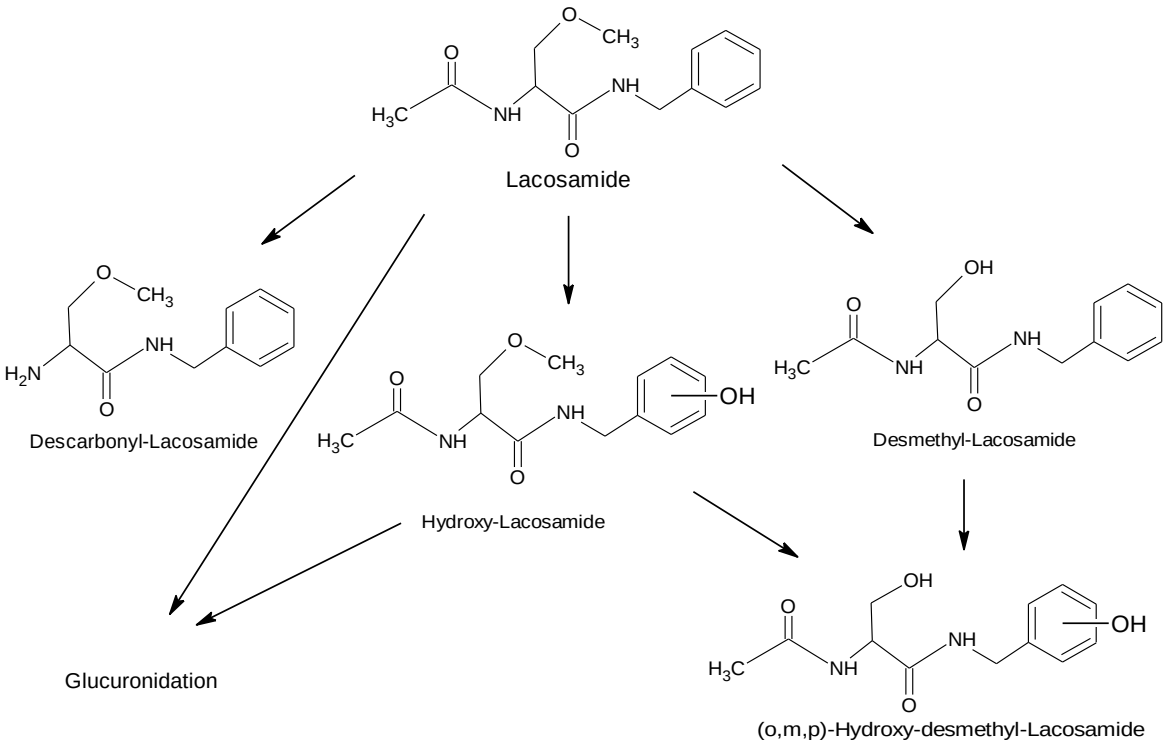


Figure 1-6: Lacosamide Metabolic Pathway.

1.6.3.5 Lamotrigine

Lamotrigine is a dichlorophenyltirazine derivative that is extensively metabolized by hepatic enzymes, mainly by conjugation with glucuronic acid (Figure 1-7). It is eliminated in the urine (8% as a parent drug and 62% as metabolites). Its major metabolites that have been detected in urine are lamotrigine-N-2-glucuronide and N-5-glucoronide (137, 140).

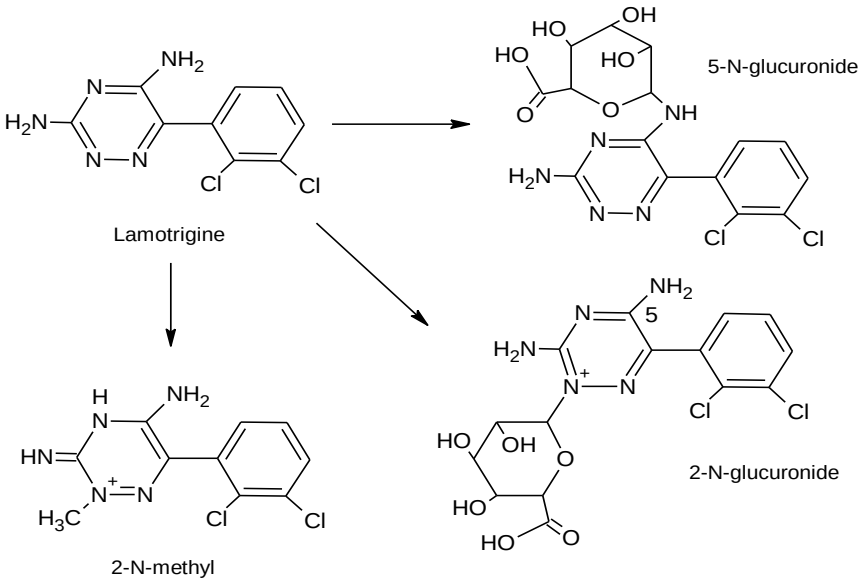


Figure 1-7: Lamotrigine Metabolic Pathway.

1.6.3.6 Levetiracetam

Levetiracetam is a pyrrolidine derivative from piracetam. Sixty six per cent of the drug is eliminated unchanged by kidney, 24% is metabolised to a carboxylic acid derivative known as L057, 2% to a pyrrolidine ring hydroxylation product and 1% to a ring fission product (Figure 1-8) (137).

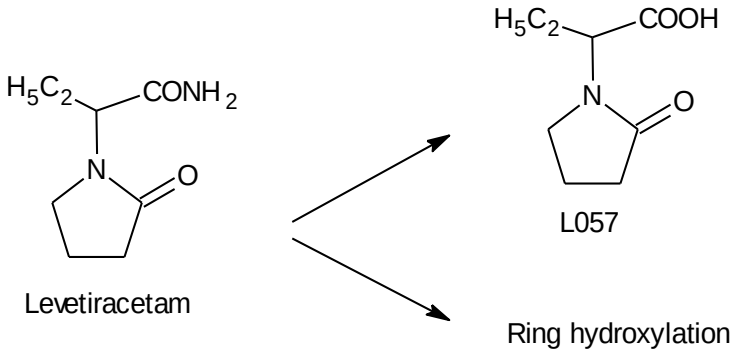


Figure 1-8: Levetiracetam Metabolism Pathway.

1.6.3.7 Phenobarbital

Phenobarbital is a barbituric acid derivative that is mainly metabolized by N-glucoside formation and by oxidation to p-hydroxy phenobarbital which in turn conjugates with glucuronide acid (Figure 1-9). The drug is excreted in urine as free drug (25-33%), N-glucosyl phenobarbital (24-30%) and free or conjugated p-hydroxy phenobarbital (18-19%)(137) .

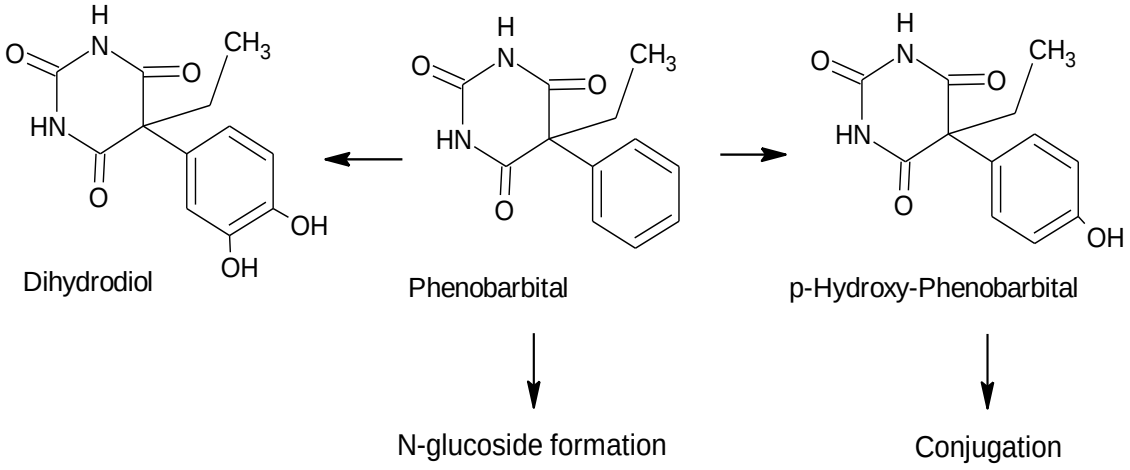


Figure 1-9: Phenobarbital Metabolic Pathway.

1.6.3.8 Phenytoin

Phenytoin is a diphenyl hydantoin analogue metabolized by p-hydroxylation of the phenyl ring and formulates the known metabolite, HPPH (p-hydroxyphenytoin) which accumulates in the plasma in concentration as high as 37 mg/l. Other products of oxidation are m-hydroxylated and 3,4-dihydroxyphenyl derivatives which appear as minor metabolites (Figure 1-10). The urinary excretion profile shows > 4% as unchanged drug while the p-hydroxy phenytoin as a conjugate metabolite accounts for 23-67% (137).

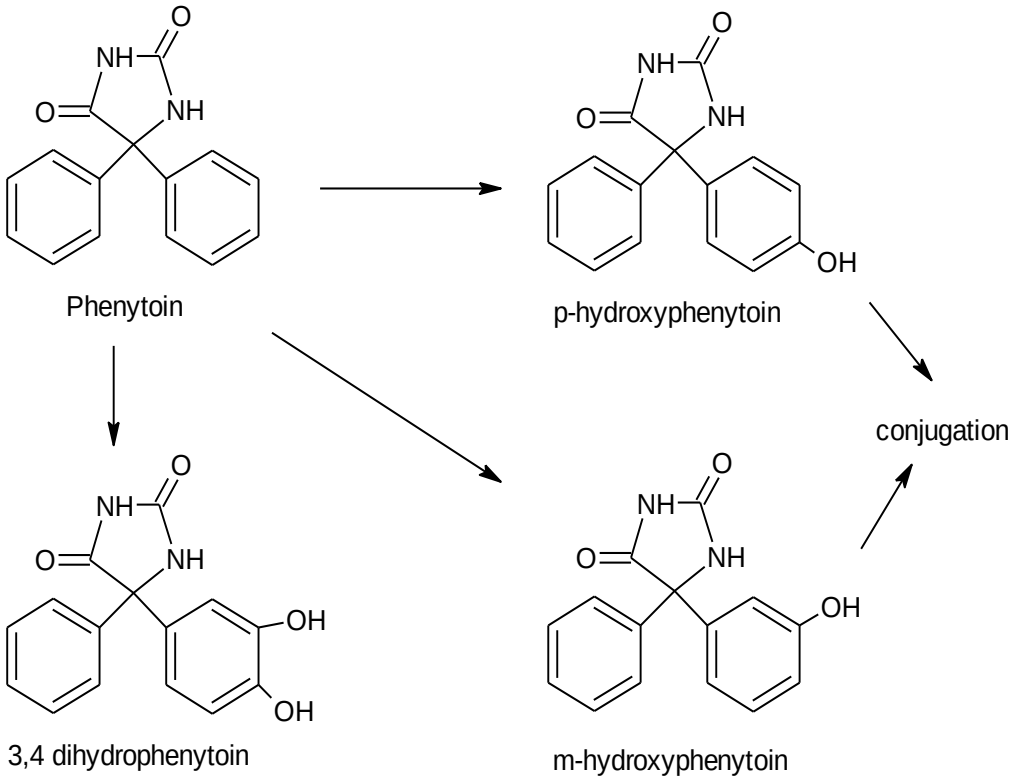


Figure 1-10: Phenytoin Metabolic Pathway.

1.6.3.9 Pregabalin

Pregabalin is a gamma amino butyric acid (GABA) and gabapentin analogue. Ninety two per cent of the drug is eliminated unchanged in urine, 0.9% is eliminated as N-methyl pregabalin and 0.4 % as unidentified metabolite (Figure 1-11) (137).

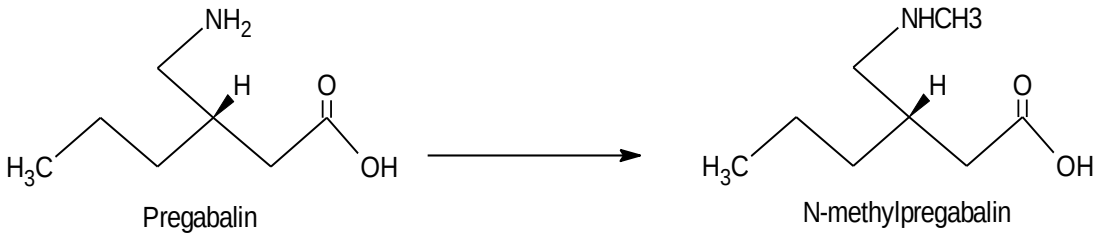


Figure 1-11: Pregabalin Metabolic Pathway.

1.6.3.10 Rufinamide

Rufinamide is a triazole derivative that is extensively hydrolysed to inactive metabolites; CGP 47292 that undergoes glucuronidation and a carboxylic derivative. Only 2% of the drug is eliminated unchanged in urine whereas 66% is eliminated as free or conjugated CGP47292 (Figure 1-12) (137).

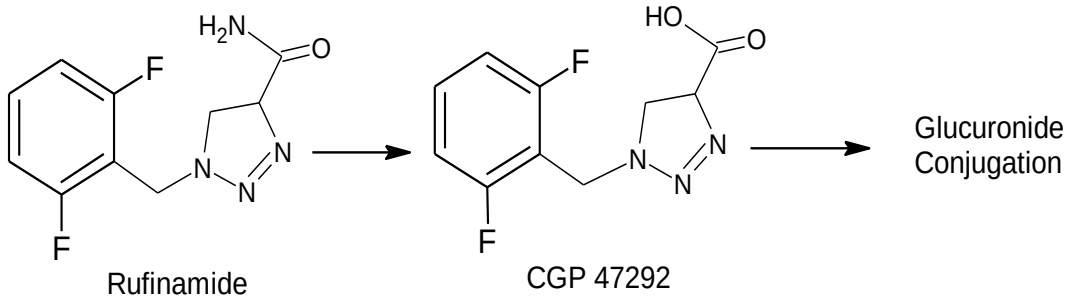


Figure 1-12: Rufinamide Metabolic Pathway.

1.6.3.11 Retigabine

Retigabine is predominantly metabolized via N-glucuronidation and N-acetylation that results in the formation of two distinct inactive N-glucuronides and an N-acetyl metabolite that demonstrates minimal pharmacologic activity. The majority of drug and metabolite are renally excreted. Retigabine is not metabolized via CYP isoenzymes (Figure 1-13) (130).

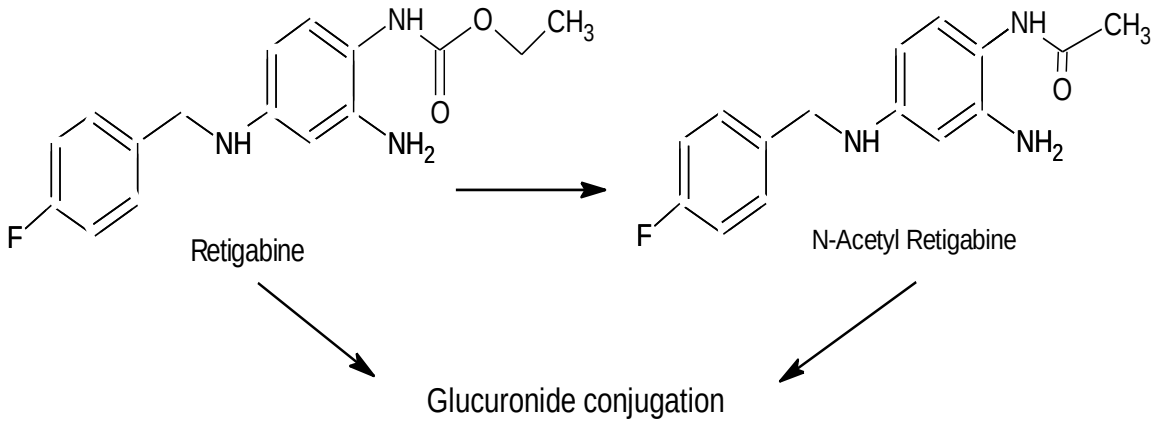


Figure 1-13: Retigabine Metabolic Pathway.

1.6.3.12 Stiripentol

Stiripentol is metabolized by O-dealkylation of the methylenedioxy moiety, resulting in the corresponding di-hydroxy analog (Figure 1-14). This dihydroxy intermediate is then methylated at either hydroxy position by the catechol 0-methyl transferase system giving p- and m-hydroxy metabolites which are urinary eliminated by conjugation (141).

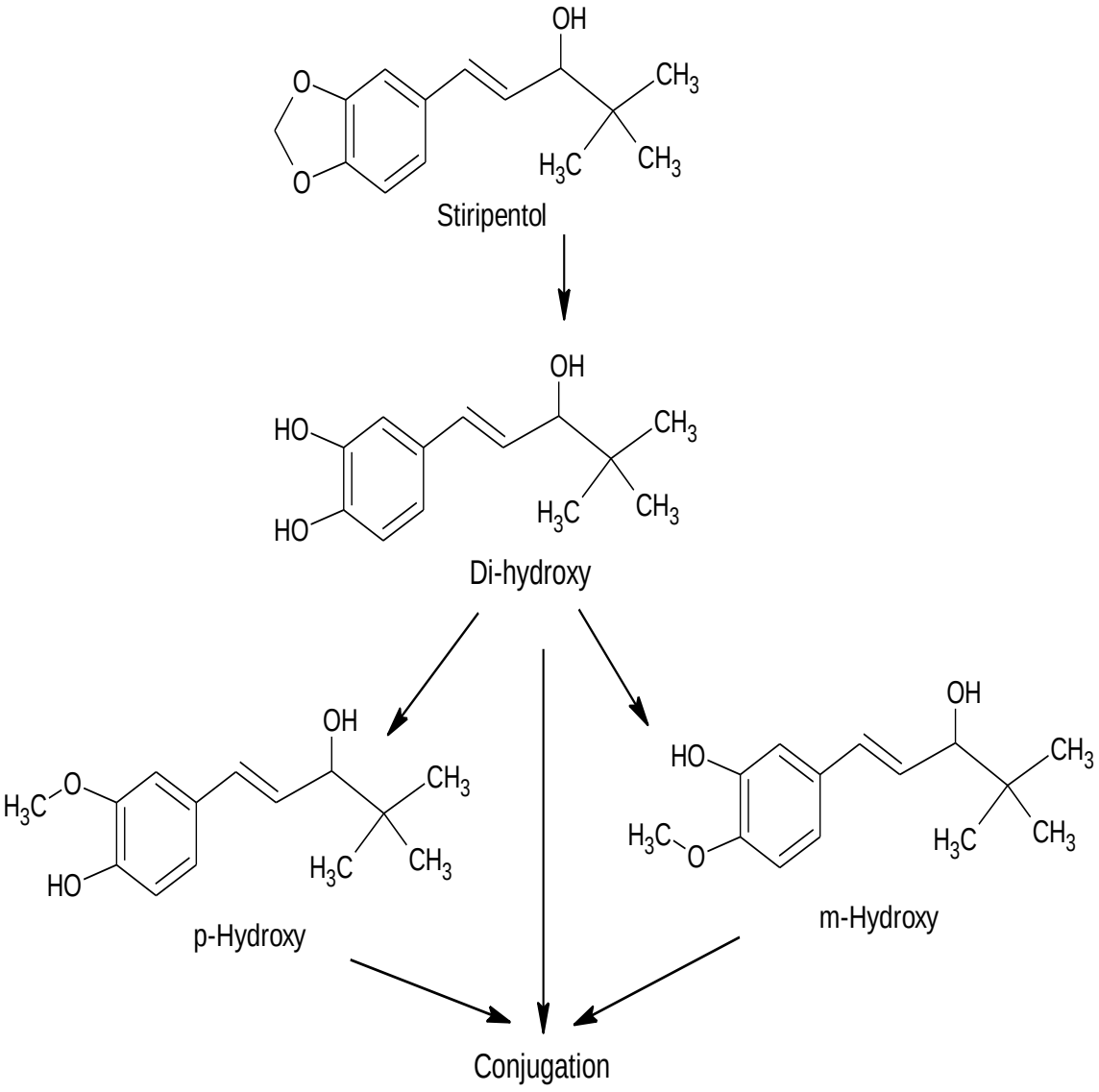


Figure 1-14: Stiripentol Metabolic Pathway.

1.6.3.13 Tiagabine

Tiagabine is a GABA-reuptake inhibitor metabolized extensively by thiophene ring oxidation and glucuronidation (Figure 1-15). It is eliminated by urine (25%) and faeces (63%) and only 2% is eliminated unchanged (137) .

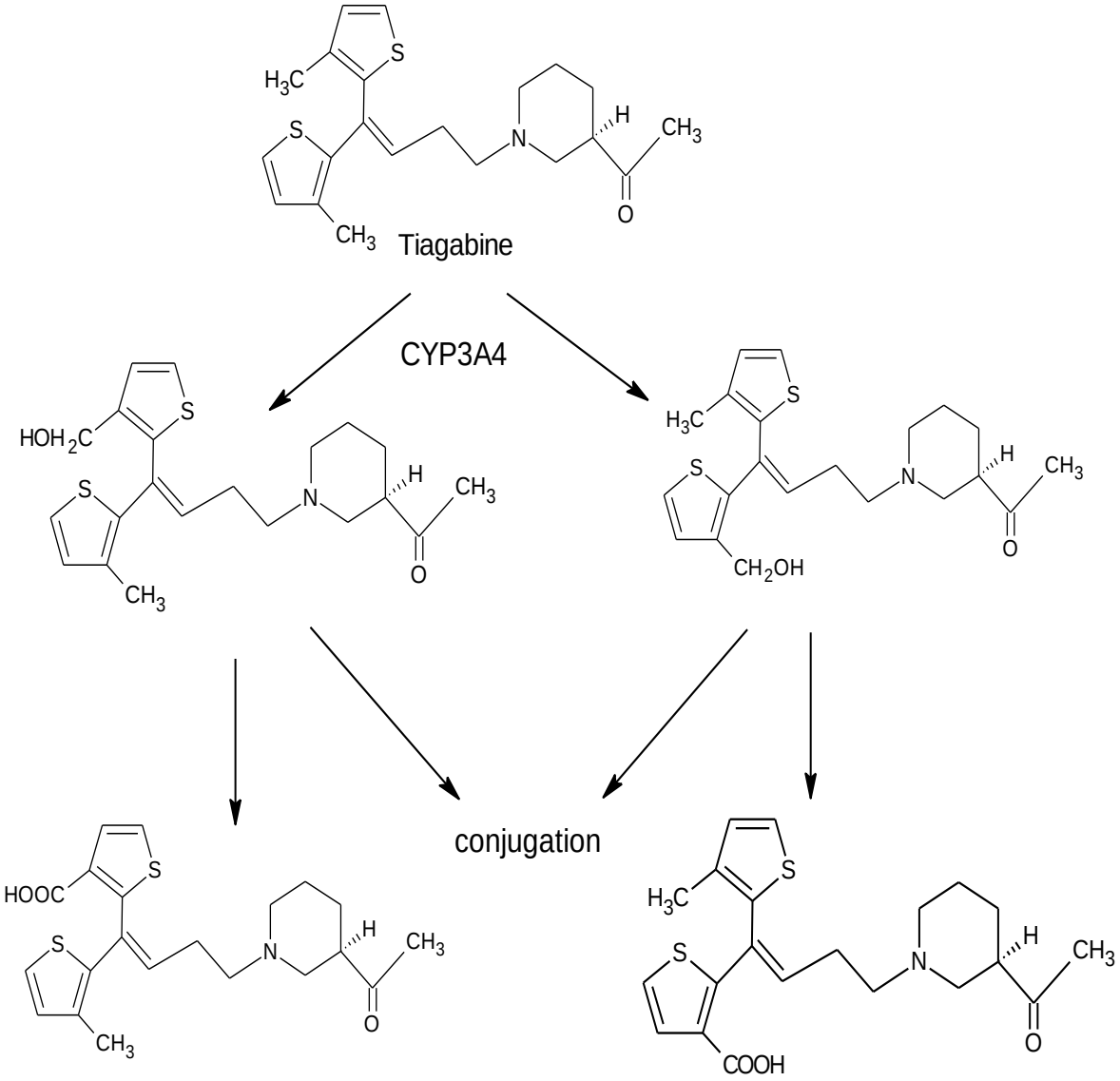


Figure 1-15: Tiagabine Metabolic Pathway.

1.6.3.14 Topiramate

Topiramate is a sulfamate-substituted monosaccharide. This drug is eliminated mainly in urine (81%). Forty per cent of the drug is eliminated unchanged. The rest is metabolized to formulate six metabolites which result from C-oxidation and their glucuronide or sulphate conjugates (Figure 1-16). The major two metabolites are 2, 3-di-OH-topiramate and 10-OH- topiramate (137).

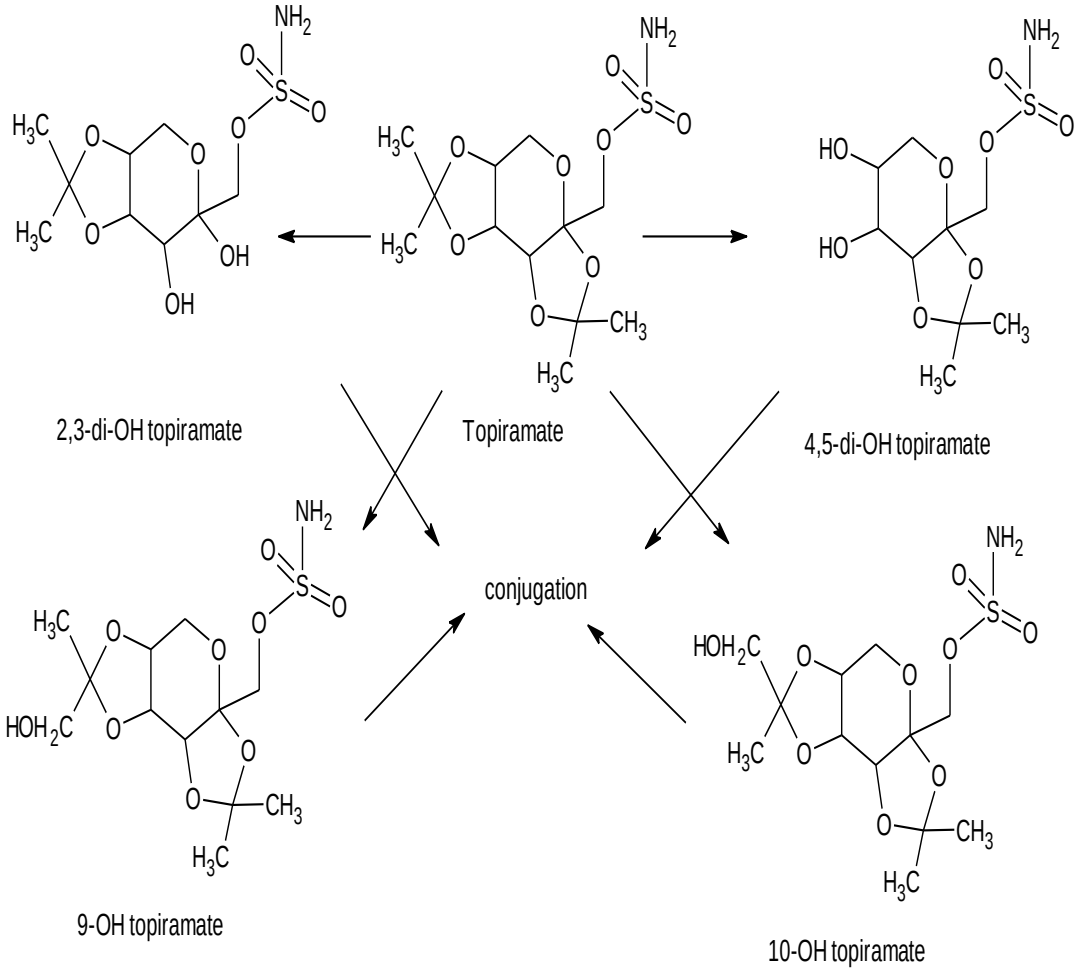


Figure 1-16: Topiramate Metabolic Pathway.

1.6.3.15 Valproic acid

Valproic acid is a carboxylic acid derivative, 59% of the parent drug is eliminated as a glucuronide conjugate and 23% as 3-ketovalproic acid in addition to other minor metabolites and products of valproic acid desaturation which are found in plasma (Figure 1-17) (137).

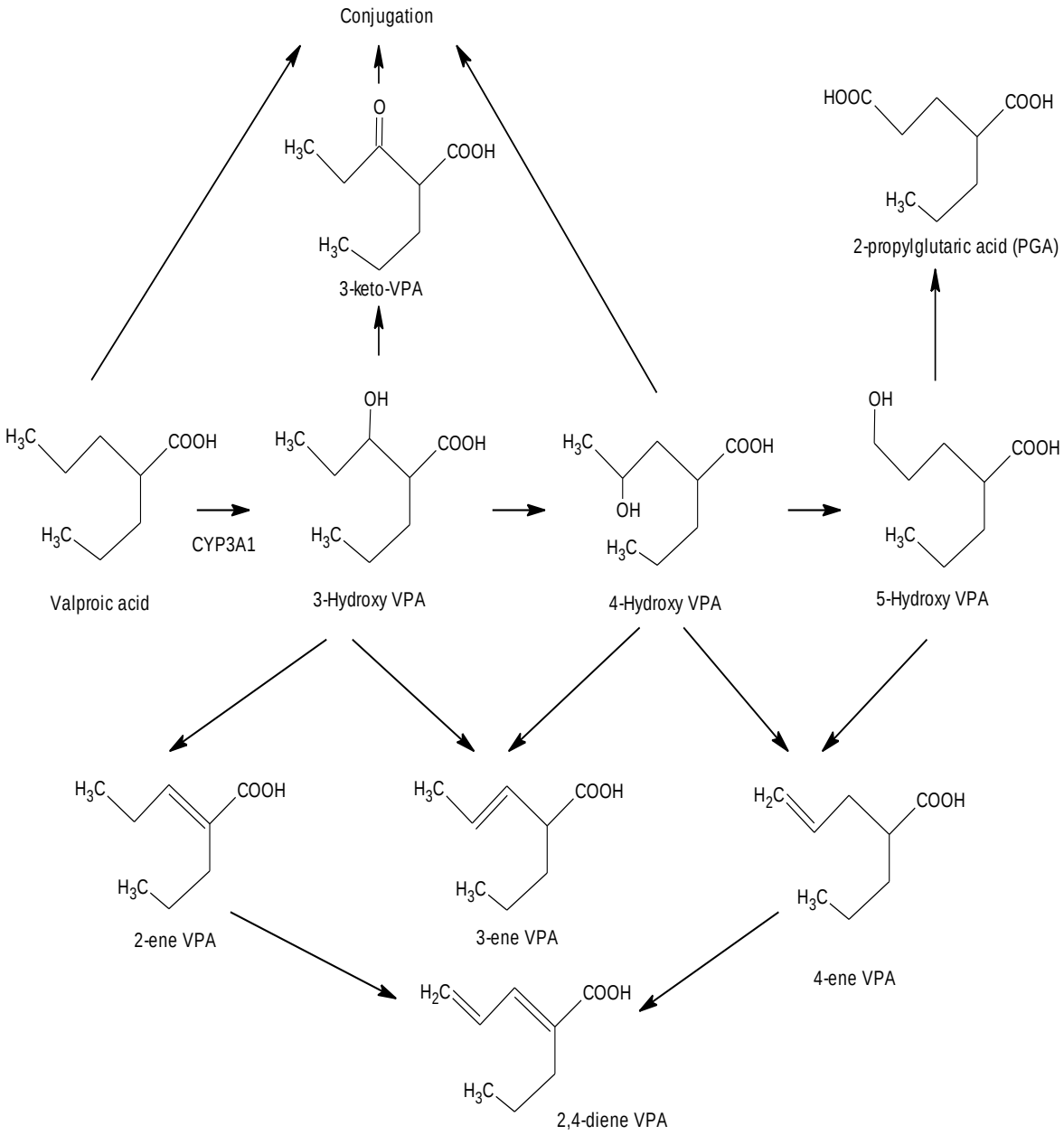


Figure 1-17: Valproic acid Metabolic Pathway.

1.6.3.16 Vigabatrin

Vigabatrin is a structural analogue of GABA that is eliminated mainly unchanged (82%). Two urinary metabolites have been detected but they have been found to represent less than 5% of a single dose (137).

1.6.3.17 Zonisamide

Zonisamide is a sulphonamide derivative with nonlinear plasma kinetics when the dose exceeds 400 mg. It is slowly eliminated in the urine (63%). Twenty two per cent of the drug is eliminated unchanged, 9.3% as N-acetylzonisamide and 3% as a glucuronide ring fission product known as M1 (Figure 1-18) (137).

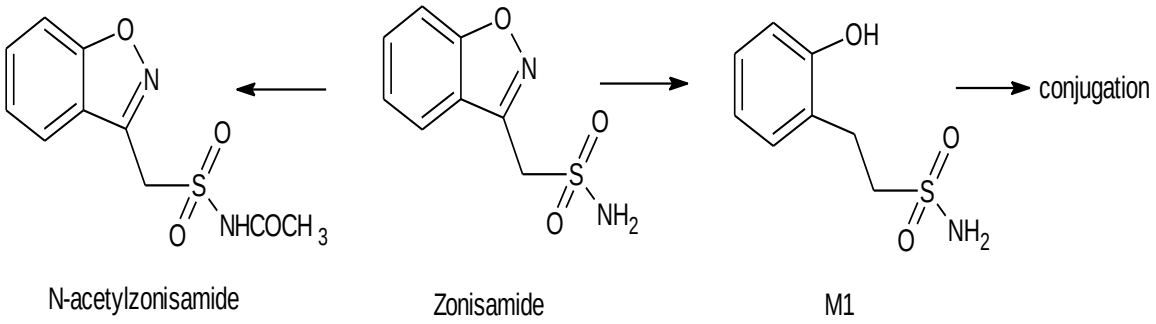


Figure 1-18: Zonisamide Metabolic Pathway.

1.6.4 Antiepileptic Drug Interactions

Pharmacokinetic interactions result from any alteration in the drug absorption, protein binding, and metabolism and excretion pathway. The most common interactions are due to hepatic enzyme induction or inhibition, mainly Cytochrome P450 enzyme (CYP). This enzyme has a number of isoenzymes that has its unique gene code and characteristic substrate specificity. Knowledge of the isoenzymes involved in the metabolism of established AEDs allows a prediction of interactions with new drugs in development. CYP isoenzymes involved in AEDs interactions are CYP1A2, CYP2C9/10, CYP2C19, and CYP3A3/4. Another key enzyme is uridine glucuronyl transferases (UGTs) which catalyse glucuronidation via two enzyme families, UGT1 and UGT2. UGTs are in general less substrate specific compared to CYP. As for the CYPs, UGTs are susceptible to induction or inhibition but their role in AED metabolism is still under investigation.

Antiepileptic drugs (AEDs) are a group of drugs that are highly susceptible to drug interactions because of their effect on CYP and UGT enzymes or for being a substrate for these two enzymes. For instance, some of the older AEDs (such as carbamazepine, phenobarbital, phenytoin and primidone) cause CYP and/or UGT induction resulting in a decrease in the serum concentration of other prescribed drugs. On the other hand, other AEDs cause CYP and/or UGT inhibition (valproic acid) resulting in an increase in the serum concentration of other medications (e.g. oral contraceptives, antidepressants, antipsychotics, anticoagulants, antimicrobal drugs, antineoplastic drugs, and immunosuppressants). Newer AEDs involved in pharmacokinetic interactions include felbamate, rufinamide, and stiripentol as enzyme inhibitors and oxcarbazepine and topiramate as enzyme inducers. Conversely, the serum concentrations of AEDs may be increased by enzyme inhibitors among antidepressants and antipsychotics, antimicrobal drugs (as macrolides or isoniazid) and decreased by other mechanisms as enzyme induction, reduced absorption or excretion such as oral contraceptives, cimetidine,

probenicid and antacides (130, 142). Table 1-5 summarise the effect of AEDs on CYP and UGT isoenzymes and the AEDs that play as a substrate for these enzymes.

Table 1-5: AEDs Mechanisms of Elimination and Susceptibility to Pharmacokinetic Interactions.

AED	Main Route of Elimination	CYP* Metabolism Pathway	AED Effect(s) on CYP	UGT* Metabolism Pathway	AED Effect on UGT
Carbamazepine	Oxidation	Yes	Inducer	No	Inducer
Eslicarbazepine acetate	Oxidation	No	CYP3A4 inducer	Yes	None
Gabapentin	Renal excretion	No	None	No	None
Lacosamide	Demethylation	No	None	No	None
Lamotrigine	Conjugation	No	None	Yes	None
Levetiracetam	Hydrolysis, renal excretion	No	None	No	Unknown
Oxcarbazepine	Conjugation, renal excretion	No	CYP3A4 inducer & CYP2C19 inhibitor	Yes	Inducer
Phenobarbital	Conjugation, renal excretion	Yes	Inducer	Yes	None
Phenytoin	Oxidation	Yes	CYP3A4 inducer & CYP2C19 inhibitor	No	Inducer
Pregabalin	Renal excretion	No	None	No	None
Rufinamide	Hydrolysis, glucuronidation	No	CYP3A4 inducer	Yes	None
Retigabine	Glucuronidation, acetylation	No	None	No	None
Stiripentol	Oxidation, glucuronidation	No	Inhibitor	No	None
Tiagabine	Oxidation	Yes	None	No	None
Topiramate	Oxidation, renal excretion	Yes	CYP3A4 inducer & CYP2C19 inhibitor	No	None
Valproic acid	Oxidation, conjugation	Yes	CYP2C19 inhibitor	Yes	Inhibitor
Vigabatrin	Renal excretion	No	None	No	None
Zonisamide	Oxidation, renal excretion	Yes	None	No	None

*CYP: Cytochrome P450 enzyme, UGT: Uridine diphosphate glucuronosyltransferase enzymes.

1.6.5 AED Mechanism of Action

Epilepsy is a neurological disorder represented by different types of seizures. The development of these seizures includes many structures and neurological pathways such as

neurons, ion channels, receptors, ganglia, and inhibitory and excitatory synapses. Generally, these seizures are induced as a result of imbalance between factors that manipulate the excitatory postsynaptic potential (EPSP) such as depolarization, sodium and calcium channels and those which manipulate the inhibitory postsynaptic potential (IPSP) such as potassium and chlorine channels (143). AEDs are developed to modify these factors by favouring inhibition over excitation in order to stop the seizure activity. Mechanisms of action of the most commonly used AEDs are illustrated in Figure 1-19.

Regarding their major mechanisms of action, AEDs can be classified into (5, 6, 144, 145):

A. Sodium channel blockers: carbamazepine, eslicarbazepine acetate, oxcarbazepine, lacosamide, lamotrigine, phenytoin, rufinamide and zonisamide.
B. Calcium channel blockers: gabapentin, pregabalin and ethosuximide.
C. Potassium channel activators: retigabine.
D. GABA enhancers: benzodiazepines, barbiturates, tiagabine and vigabatrin.
E. SV2A modulators: levetiracetam.
F. Drugs with multiple mechanisms of action: felbamate, topiramate and valproic acid.

All these drugs, chiefly the new generation drugs, have more than one mechanism of action which gives them a wide range of neurological effects that lead to their off-label use in many disorders. Table 1-6 demonstrates the different mechanisms of action related to these drugs. It is noticeable that most of these drugs have an effect on the GABA neurotransmitter, its receptor or its re-uptake mechanism. GABA is the major neurotransmitter in the CNS inhibition process which is affected by benzodiazepines, common drugs of misuse in forensic toxicology (5).

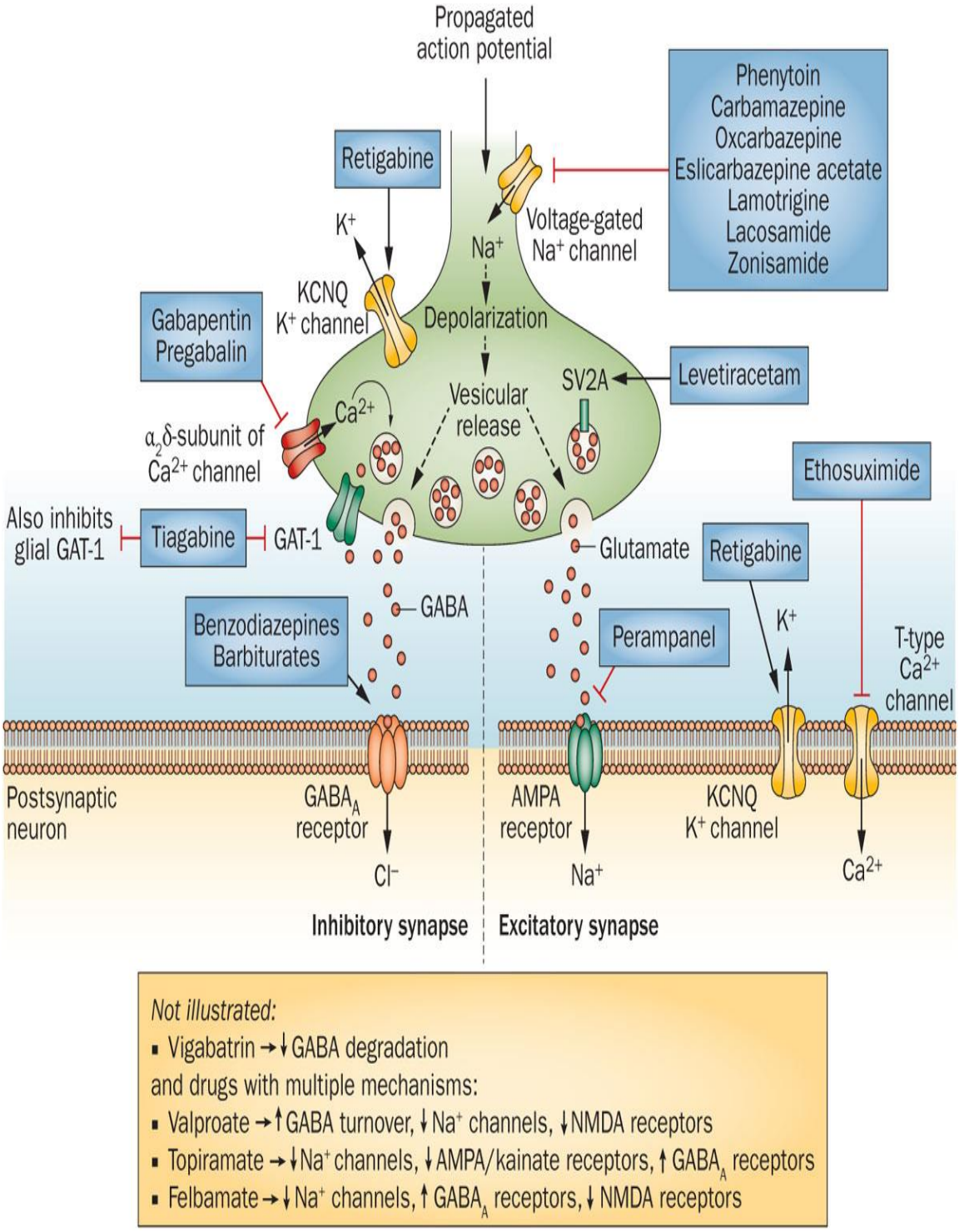


Figure 1-19: Main Mechanisms of Action of Most Commonly Prescribed AEDs (145).

"Permission Obtained From Dr. Wolfgang Loscher" (AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, GABA: γ-aminobutyric acid, GAT-1: sodium- and chloride-dependent GABA transporter 1, SV2A: synaptic vesicle glycoprotein 2A. All these factors are involved in synaptic vesicle cycling and neurotransmitter release in normal and pathological conditions).

Table 1-6 : AED Mechanism of Action (5, 6, 130, 132, 146, 147).

Drug	Na^+ channel inhibitors	Ca^{+2} channels inhibitors	K^+ channel activators	GABA enhancers	Glutamate inhibitors	SV2A modulators
Carbamazepine	●			●	●	
Eslicarbazepine	●			●	●	
Gabapentin		●		●		
Lacosamide				●	●	
Lamotrigine	●	●				
Levetiracetam		●		●	●	●
Oxcarbazepine	●			●	●	
Pregabalin		●		●		
Phenobarbital				●	●	
Phenytoin	●					
Retigabine			●			
Rufinamide	●					
Stiripentol				●		
Tiagabine				●		
Topiramate	●			●	●	
Valproic acid	●	●		●	●	
Vigabatrin				●		
Zonisamide	●	●		●		

1.7 AEDs Indications

The major indication for which AEDs are manufactured is epilepsy disorder. Carbamazepine and valproate are licensed for use in all type of epilepsy seizures. Stiripentol is the drug of choice in children with epilepsy. Due to the side effects of retigabine; blue-grey discoloration of ocular tissue, nails, lips and skin, it is only recommended for use in cases of drug-resistant seizures. Most AEDs are licensed for mono or adjunctive therapy for the treatment of focal seizures with or without secondary generalised seizures. Focal seizures are those which start in one part of the brain. When they spread to the rest of the brain causing generalised seizures this is known as secondary generalisation. Phenobarbital and phenytoin are largely used intravenously in status epilepsy where seizures last for more than 30 minutes and become life threatening that emergency treatment is needed. Levetiracetam is licensed for use in juvenile myoclonic epilepsy; a type that experiences seizures associated with muscle twitching or jerking. Finally rufinamide followed by lamotrigine are drugs of choice in cases of Lennox-Gastaut syndrome (148). Lennox-Gastaut syndrome is an uncommon and difficult to treat type of childhood epilepsy that occurs in between one to five in every 100 children with epilepsy. Usually, it starts between three and five years of age and is associated with learning difficulties.

Generally, 18% of epileptic patients are using a polytherapy of AEDs to get an acceptable control of their seizures. The most frequently combinations of AEDs are lamotrigine with valproate (42%), phenobarbital with phenytoin (20%), and carbamazepine with levetiracetam (19%) (149). As a result of their multiple targets of action, many preclinical studies suggest that these drugs could be effective in other CNS disorders beside their use in epilepsy. The extensive use of AEDs to treat non-epilepsy disorders, both in neurology and psychiatry was discussed earlier (see 1.4.1). In the UK, carbamazepine is licensed for use in manic psychosis and trigeminal neuralgia. Gabapentin is approved for neuropathic pain and migraine but is being prescribed off label for migraine prophylaxis. Pregabalin is also licensed to treat neuropathic pain in addition to generalized anxiety disorder. Lamotrigine and valproate are being used in depression associated with bipolar disorder and finally topiramate is approved for migraine prophylaxis (Table 1-7).

Table 1-7: AED Trade Name, Daily Dose and Licensed Indications in the UK and Their Label Prescription in Epilepsy and Non-epilepsy Disorders (148).

Generic name	Trade name	UK licence	Licensed indication in epilepsy	Licensed in other indications	Maintenance dose (mg/day)
Carbamazepine	Tegretol	1965	All forms of epilepsy	Prophylaxis of manic depressive-psychosis in patient unresponsive to lithium therapy, trigeminal neuralgia	400-1600
Eslicarbazepine acetate	Zebinix	2009	Adjunctive treatment in adults with focal seizures with or without secondary generalisation	None	400-800
Gabapentin	Neurontin	1993	Monotherapy and adjunctive treatment of focal seizures with or without secondary generalisation	Neuropathic pain, migraine prophylaxis (unlicensed)	100-4800
Lacosamide	Vimpat	2008	Adjunctive treatment of focal seizures with or without secondary generalisation	None	100-800
Lamotrigine	Lamictal	1991	Monotherapy and adjunctive treatment of focal seizures and generalised seizures, Lennox-Gastaut syndrome	Prevention of depressive episodes associated with bipolar disorder	100-700
Levetiracetam	Keppra	2000	Monotherapy and adjunctive treatment of focal seizures with or without secondary generalisation, juvenile myoclonic epilepsy	None	1000-3000
Oxcarbazepine	Trileptol	2000	Monotherapy or adjunctive therapy of focal seizures with or without secondary generalised seizures	None	1200-1400
Phenobarbital	Phenobarbital	1912	Status epilepticus	None	10 mg/kg
Phenytoin	Epanutin	1938	Status epilepticus; acute symptomatic seizures associated with head trauma or neurosurgery	None	300-400
Pregabalin	Lyrica	2004	Adjunctive therapy for focal seizures with or without secondary generalisation	Neuropathic pain, generalized anxiety disorder	50-200

Table 1-7: AED Trade Name, Daily Dose and Licensed Indications in the UK and Their Label Prescription in Epilepsy and Non-epilepsy Disorders (Continued…).

Generic name	Trade name	UK licence	Licensed indication in epilepsy	Licensed in other indications	Maintenance dose (mg/day)
Retigabine	Trobalt	2011	Drug-resistant focal seizures	None	600-1200
Rufinamide	Inovelon	2007	Adjunctive treatment of seizures in Lennox-Gastaut syndrome	None	200-1600
Stiripentol	Diacomit	2006	Orphan drug for severe myoclonic epilepsy of infancy	None	10-50 mg/kg
Tiagabine	Gabitril	1998	Adjunctive treatment for focal seizures with or without secondary generalisation	None	4-32
Topiramate	Topamax	1995	Monotherapy or adjunctive in generalised or focal seizures with or without secondary generalisation	Prophylaxis of migraine	200-1600
Sodium valproate	Epilim	1973	All forms of epilepsy	Semi-sodium valproate for acute manic episode associated with bipolar disorder	250-2500
Valproic acid	Depakote, Convulex	1993	All forms of epilepsy	Acute mania associated with bipolar disorder	250-2500
Vigabatrin	Sabril	1973	Monotherapy or adjunctive treatment in focal epilepsy with or without secondary generalisation	None	1000-4000
Zonisamide	Zonergan	2005	Adjunctive treatment in focal seizures with or without secondary generalisation	None	100-400

1.8 AEDs Side Effects and Overdose Symptoms

With respect to their multiple mechanisms of action, AEDs are reported to have undesirable side effects on the central nervous system. Somnolence, dizziness and ataxia are common side effects of all AEDs (150) in addition to the increased risk of imbalance within the therapeutic dose (151). All AEDs in all dose-analysis studies demonstrated that they increase the risk of imbalance and ataxia compared with placebo suggesting an overall dose-response effect in both old and new generation drugs (151). The major adverse effects of concern are related to action on the mental functions which present as cognitive impairment and psychological and behavioural disturbances (152) The most frequent cognitive effects seem to be sedation, amnesia, confusion, blurred speech, abnormal thinking, and depression (153). Some drugs also cause euphoria (93, 99, 105, 153). A summary of these effects is demonstrated in Table 1-8.

Cognitive impairments include delirium, memory disturbances (amnesia, fugue) as well as effects on intellect and behaviour. AEDs are classified among drugs that induce cognitive impairment owing to their effects on reducing neural irritability which leads to an increase in memory deficits and psychomotor slowing. Most of them potentiate the effect of GABA, the major inhibitory neurotransmitter. In turn, GABA impairs the function of the subcortical structures which play an important role in the consolidation of newly learned information. Thus, AEDs which affect GABA receptors may impair potentiation and cause deficits both in the acquisition and the retention of information. This effect is dose related and reversible after reducing the dose of the AED (154). Frequency and severity of cognitive impairment resulting from the use of AEDs has been correlated with the pharmacokinetics and pharmacodynamics (such as elimination half-life and receptor binding affinity) in addition to the duration of the drug administration (154, 155).

Table 1-8: AED Mental and Neurological Side Effects and Overdose Symptoms.

Drug name	Mental side effects	Overdose effects	References
Carbamazepine	Amnesia, ataxia, unsteadiness, psychotic symptoms, phobias, mania	Coma, convulsions, respiratory depression and death	(148, 153)
Eslicarbazepine acetate	Drowsiness, headache, impaired coordination, confusion, mood changes, psychosis, impaired memory, suicidal ideation	No cases have been reported	(148)
Gabapentin	Sedation, amnesia, ataxia, paraesthesia	Sedation, tachycardia, hypotension	(153, 156, 157)
Lacosamide	Memory and cognition impairment, somnolence, blurred vision	Coma, respiratory depression, status epilepticus	(46)
Lamotrigine	Memory loss, sedation, cognitive impairment, ataxia, confusion	Convulsions, respiratory depression	(35, 153, 157)
Levetiracetam	Sedation, amnesia, agitation, irritability, psychosis	Hallucination, respiratory depression	(153, 156, 157)
Oxcarbazepine	Ataxia, tremor, vertigo, somnolence	Sedation, ataxia, nystagmus	(153, 156, 157)
Phenobarbital	Drowsiness, lethargy, depression, ataxia, paradoxical excitement, hallucinations, impaired memory and cognition	Coma, hypotension, respiratory depression	(153, 158)
Phenytoin	Slurred speech, ataxia, confusion	Respiratory depression	(153)
Pregabalin	Sedation, disturbed attention and memory loss, confusion, paraesthesia, euphoria	Sedation, tachycardia, hypotension.	(153, 157)

Table1-8: AED Mental and Neurological Side Effects and Overdose Symptoms (Continued…).

Drug name	Mental side effects	Overdose effects	References
Retigabine	vertigo, amnesia, paraesthesia, impaired coordination, speech and attention, confusion, psychosis, anxiety, suicidal ideation	Discoloration of ocular tissue, nails, lips and skin, cardiac arrhythmia (cardiac arrest)	(148, 159)
Rufinamide	Drowsiness, insomnia, anxiety, impaired coordination, depression, agitation, and activation of suicidal ideation	No cases have been reported	(148, 160)
Stiripentol	Aggression, anorexia, ataxia, drowsiness, sleep disorders	No cases have been reported	(148)
Tiagabine	Somnolence, sedation, confusion, psychosis, impaired concentration	Coma, respiratory depression, status epilepticus	(44, 153, 157)
Topiramate	Sedation, amnesia, drowsiness, confusion, somnolence, psychomotor retardation	Memory loss, sedation, coma and death. It cause the greatest impairment of neuro-cognitive impairment	(153, 156, 157, 161)
Valproic acid	Sedation, lethargy, confusion, reversible dementia	Encephalopathy, coma	(153)
Vigabatrin	Ataxia, paraesthesia, impaired concentration, confusion, depression	Visual defects	(153, 161)
Zonisamide	Psychiatric changes, somnolence, reduced concentration	Memory loss, coma, speech difficulties, respiratory depression and death	(49, 153, 157)

Over 50% of reported AED overdoses are considered as a consequence of the new generation drugs. In most cases of AED overdose, the drugs produce some degree of CNS impairment at low toxic doses, while at higher toxic doses; coma, seizures and death have been reported as a result of respiratory depression or cardiac disturbances. In addition, each drug has other specific poisoning effects related to its mechanism of action (156). Some AEDs (carbamazepine, lacosamide, lamotrigine, oxcarbazepine, phenobarbital, phenytoin, primidone, and rufinamide) cause what is so-called AED hypersensitivity syndrome which is a fatal syndrome. The symptoms usually start between 1 and 8 weeks of exposure including fever, rash, and lymphadenopathy. Other systemic signs are liver dysfunction, haematological, renal, and pulmonary abnormalities, vasculitis, and multi-organ failure (148).

1.9 AEDs Analysis- A Review of Previous Work

1.9.1 AEDs and Biological Matrices

Multiple analytical methodologies have been reported for the measurement of all AEDs in plasma and serum for therapeutic drug monitoring (TDM) but less information has been found on the detection of the new generation of AEDs in whole blood and urine (162-164). Few articles have mentioned the analysis of conventional AEDs in hair (67, 165-169). However, no studies were found regarding the analysis of the newer AEDs in hair. Only one paper published in 2014 analysed pregabalin in hair (170). All these studies used hair for TDM purposes. AEDs analysed were carbamazepine, phenytoin, lamotrigine, valproic acid and pregabalin in human hair in addition to phenobarbital in rat hair (169-174).

Blood and urine are the conventional specimens used to document drug facilitated crime (DFC). Blood, plasma and serum are the samples of choice for detecting drugs in general owing to the best correlation between the effects of drugs and their concentrations in these biological fluids, if equilibrium distribution between all tissues has been reached (175). The next favorable sample is urine. Collection of urine samples does not require medical supervision but it should be collected at the earliest opportunity especially if a rapidly eliminated drug is suspected to have been used. Urine is the most useful sample to be taken in DFC cases as it gives a much greater chance of drugs being detected. It is also essential if the full range of relevant toxicology tests are to be carried out (88).

However, when blood and urine samples are not available (not sampled, degraded, lost, crime not reported at the time of DFC), hair as a sample represents the best alternative choice for analysis (176). Hair has been considered as a valuable specimen in situations such as DFC for many reasons. Firstly, in DFC, there is often a delay in reporting the crime, so the natural processes have had enough time to eliminate the drug from typical biological specimens (blood, urine). Hair samples are found to have a large surveillance window (weeks to months depending on the length of the hair shaft) compared to 2-4 days for most drugs in urine and blood (91). Secondly, hair samples are easier to collect from the victim and the embarrassment associated with urine collection especially in case of sexual assault can be greatly mitigated (177). Thirdly, hair analysis has the ability to distinguish between chronic use and single dose exposure, while urine and blood analysis is unable to make this distinction (91, 176). This unique property of hair testing is very important both in the interpretation of results in cases of taking hypnotic drugs concomitantly for therapeutic reasons and in the discrimination of false assault reports such as revenge cases where little evidence is available. The victim could claim that the defendant has spiked his/her drink with a drug (177) whereas in reality the victim him/herself has abused this drug for a long time. In such cases, hair testing only can discriminate between chronic use and single dose.

Generally, for practical purposes, the two tests are applied as they are complementary to each other. Urine and blood analysis provides short term information of an individual's drug use, whereas long term histories are accessible through hair analysis (91, 176).

Another concern in forensic work is in relation to the stability of substances in human samples. Samples should be stable and preserve the analytes as long as possible. For this reason, analyzing human hair has become an important tool next to blood and urine analysis on the grounds that the hair samples are stable for a long time and the majority of drugs and their metabolites can be analyzed years after drug incorporation into the hair. Taking into account these assets, this research will evaluate different types of human samples including blood, urine and hair and establish a method to detect the drugs of interest which have the highest incidence of abuse such as gabapentin and pregabalin.

1.9.2 Sample Extraction Techniques of AEDs

The separation process of most AEDs from biological samples relies on protein precipitation with acetonitrile or methanol (178). Liquid-liquid extraction (LLE) is

sometimes required after the deproteinization step (179). LLE has been the method of choice for years however the technique suffers from several disadvantages (180). These include problems of environmental concern due to high levels of solvent usage, difficult automation and poor extraction recoveries for some drugs. Solid phase extraction (SPE) has been used to overcome the disadvantages of LLE (181).

SPE allows the use of lower sample volumes and lower solvent volumes with shorter sample extraction times. Biological samples are notoriously dirty; injecting them with minimum clean up onto very sensitive and expensive instruments makes very little sense. SPE has been shown to significantly increase gas (GC) and liquid chromatography (LC) column life while reducing the downtime on equipment for source cleaning compared to LLE and protein precipitation. LLE often requires several extraction steps to achieve sample clean up. However, the more extractions required, the more drug that will be lost. LLE often has trouble achieving high recoveries on a reliable, reproducible level. SPE, on the other hand, concentrates the sample on the column and allows for reproducible results at very low concentrations of analytes. LLE and protein precipitation are general techniques that extract many compounds, whereas SPE gives the analyst the ability to extract a broad range of compounds with increased selectivity (182).

1.9.3 AEDs and Analytical Instrumentation

With respect to the analytical method used, various analytical tools have been developed for therapeutic drug monitoring of AEDs. Automated immunoassay methods have been the most appropriate methods for the determination of anticonvulsants during TDM since the 1980s (183, 184). Since immunoassays are not available for most of the newer AEDs (pregabalin, vigabatrin, tiagabine, lacosamide) with only one paper for gabapentin immunoassay analysis published recently (185), several chromatographic techniques are used, including HPLC and GC methods. Simple chromatographic applications such as Thin Layer Chromatography (TLC) have been developed in the past (186).

A number of simultaneous chromatographic assays for AEDs have been developed using GC (187-189) and HPLC alone, (190, 191) or coupled to an ultraviolet detector (192, 193) or an evaporative light-scattering detector, (194) in addition to fluorescence polarization immunoassay (183). The early initial simultaneous assays, from the 1980s, concentrated on separating the older AEDs such as ethosuximide, primidone, carbamazepine, carbamazepine-epoxide, phenytoin, and phenobarbital with the inclusion or removal of one

or more additional drugs or metabolites such as ethyl-phenacemide, 5-para-hydroxyphenyl-5-phenylhydantoin (p-HPPH), N-des-methyl-methsuximide, phenyl-2-ethyl-malonamide and lamotrigine (191, 195, 196). However, these methods required time-consuming and difficult extraction procedures or relatively large sample volumes (~1mL) as well as lengthy chromatographic run times that limited their throughput capacity and sensitivity (178). All methods which employed ultraviolet detection have the risk of matrix, metabolite or other co-medication interferences (197). Furthermore, a number of these previous analytical techniques are not applicable to some AEDs due to lack of volatility and chromophores for some of these such as pregabalin, gabapentin, vigabatrin, topiramate and valproic acid (198, 199).

Over the past twenty years, developed assays have focused more on separating the newer AEDs such as levetiracetam, lamotrigine, oxcarbazepine, topiramate, and zonisamide (178, 200-202). More recently, LC/MS/MS analysis has been applied to these drugs as well (179, 192, 197). These techniques have allowed for improved selectivity during separation and detection. A simultaneous LC/MS/(MS) assay would be faster and more reliable than multiple analytical assays when quantification of multiple AEDs is needed for samples of patients with poly AED therapy (197). However, few papers have been published for the simultaneous analysis of 10 or more AEDs. A simultaneous analysis of 22 AEDs by UPLC/MS/MS was reported recently, however it was for TDM purposes and samples had to undergo 3 different dilutions with 3 different calibration curves to obtain the desired results. Furthermore, it did not use any internal standard to eliminate the matrix effect associated with LC/MS/(MS) analysis (203). Another two simultaneous methods to determine 9 and 10 AEDs have also been reported. The first paper used LC/MS and it was quantitatively validated for only 6 of these drugs with their metabolites and tested the others by screening (178, 197). Few studies have been published regarding AEDs analysis in postmortem blood (171, 204, 205). The first of these included 9 AEDs and two metabolites; carbamazepine, carbamazepine-10, 11-epoxide, gabapentin, lamotrigine, levetiracetam, oxcarbazepine, 10-OH-carbazepine, phenobarbital, phenytoin, pregabalin and topiramate whereas the second one had lamotrigine, carbamazepine, 10-OH-carbazepine, phenytoin and phenobarbital only and did not cover any newer AEDs. No simultaneous studies until now have been found to include eslicarbazepine acetate, retigabine and lacosamide although individual LC/MS/MS methods on human plasma do exist for measuring these (133, 206, 207). A brief overview of simultaneous quantitative analysis methods of AEDs is presented in Table 1-9.

Table 1-9: An Overview of Studies Published for Simultaneous AED Analysis.

RF	AEDs	Sample	Sample Preparation	Instrumentation	CBZ	ESL	GBP	LAC	LEV	LTG	OXC	PHT	PBT	PGR	RTG	RUF	STP	TIG	TPR	VIG	VPA	ZNS	Others
(203), 2012	16	Plasma	PP	UPLC/MS/MS	+		+		+	+		+	+					+	+	+	+	+	CBZ-E, ETM, FBM, PRM, 6 Benzodiazepines
(178), 2010	10	Plasma	PP	LC/MS/MS	+		+		+	+	+	+							+		+	+	CBZ-E
(208), 2010	10	Plasma	PP	HPLC	+					+		+	+									+	FBM, MHD, CBZ-E, PRM
(179), 2009	11	Serum	PP, LLE	LC/MS/MS			+		+	+	+			+		+	+	+	+			+	FBM
(197), 2008	9	Plasma	SPE	LC/MS	+		+		+	+	+	+	+	+				+	+		+	+	CBZ-E, MHD, CBZ-Diol
(200), 2007	7	Serum	SPE	LC/UV	+					+	+	+	+									+	CBZ-E, CBZ-Diol, PRM
(201), 2002	6	Plasma	PP + SPE	LC/UV	+					+	+	+	+										PRM,CBZ-E, CBZ-Diol
(181), 1990	6	Serum	SPE	GC/FID	+							+	+								+		PRM, ETM
(202), 1999	6	Plasma	LLE	LC/DAD	+					+		+	+										CBZ-E,CBZ-Diol, ESM, PRM
(209), 2003	6	Plasma	-	MECC/DAD	+					+		+	+										PRM, CBZ-E
(210), 1994	5	Plasma	PP	LC/UV	+							+	+										HPPH,CBZ-E, PRM, FBM
(211), 2002	5	Plasma	SPME	GC/TSD	+					+		+											PRM, CBZ-E
(193), 2010	5	Plasma	PP	LC/UV						+	+		+									+	FBM, MHD

CBZ-E: carbamazepine-10, 11 epoxide, CBZ-Diol: carbamazepine -10-11-Diol, HPPH: 5-(4-hydroxyphenyl)-5-phenylhydantoin, MHD: 10-hydroxycarbamazepine, FBM: felbamate, PRM: primidone, ESM: ethosuximide, PP: protein precipitation, LLE: liquid-liquid extraction, FID: flame ionization detector, DAD: dual array detector, NPD: nitrogen phosphorus detector, MECC: micellar electrokinetic capillary chromatography, SPME: solid-phase microextraction, GC/TSD: gas chromatography with thermionic specific detection, CE: capillary electrophoresis, CED: coulometric electrochemical detection.

Table 1-9: An Overview of Studies Published for Simultaneous AED Analysis (Continued...).

RF	AEDs	Sample	Sample Preparation	Instrumentation	CBZ	ESL	GBP	LAC	LEV	LTG	OXC	PHT	PBT	PGR	RTG	RUF	STP	TIG	TPR	VIG	VPA	ZNS	Others
(180), 2011	5	Blood	LLE	LC/MS/MS	+					+		+	+										MHD, 12 non-AEDs
(212), 1993	4	Blood, Saliva, Urine	PP + SPE	LC/DAD	+							+	+										PRM
(213), 2002	4	Serum	-	Immunoassay	+							+	+								+		
(214), 2002	4	Serum	PP	LC/UV	+						+	+	+										CBZ-E, CBZ-Diol
(215), 2002	4	Plasma	PP	LC/UV	+						+	+	+										CBZ-E, MHD,
(192), 2005	4	Plasma	PP	LC/UV						+	+												MHD, FBM
(216), 2005	4	Serum	PP	LC/UV	+					+		+	+										
(217), 1997	3	Serum	SPE	HPLC	+					+		+											CBZ-E
(218), 2004	3	Serum	PP	LC/F			+							+						+			-
(219), 2007	3	Serum	-	Immunoassay	+							+									+		-
(220), 2007	3	Plasma	PP	LC/UV						+	+											+	MHD
(221), 2008	3	Plasma	LLE	LC/UV	+							+	+										CBZ-E
(198), 2008	3	Serum	PP	LC/MS			+							+						+			-

CBZ-E:carbamazepine-10,11 epoxide, CBZ-Diol: carbamazepine-10-11-Diol, HPPH: 5-(4-hydroxyphenyl)-5-phenylhydantoin, FBM: felbamate, MHD: 10-hydroxycarbamazepine, PRM: primidone, ESM: ethosuximide, P.P: protein precipitation, LLE: liquid-liquid extraction, FID: flame ionization detector, DAD: dual array detector, NPD: nitrogen phosphorus detector, MECC: micellar electrokinetic capillary chromatography, SPME: solid-phase microextraction, GC/TSD: gas chromatography with thermionic specific detection, CE: capillary electrophoresis, CED: coulometric electrochemical detection.

Table 1-9: An Overview of Studies Published for Simultaneous AED Analysis (Continued...).

RF	AEDs	Sample	Sample Preparation	Instrumentation	CBZ	ESL	GBP	LAC	LEV	LTG	OXC	PHT	PBT	PGR	RTG	RUF	STP	TIG	TPR	VIG	VPA	ZNS	Others
(222), 2010	3	Plasma	SPE	LC/F			+												+	+			-
(223), 1990	2	Serum	LLE	LC/UV	+							+											-
(224), 1994	2	Serum	-	Immunoassay	+							+											-
(225), 1995	2	Serum	LLE	LC/UV	+						+												6 metabolites
(226), 1996	2	Serum	PP	HPLC			+													+			-
(227), 1997	2	Serum	PP	GC/MS	+					+													CBZ-E
(228), 1998	2	Plasma	PP	LC/UV	+							+											CBZ-E
(229), 1998	2	Urine/Serum	PP	LC/F			+													+			-
(230), 2003	2	Plasma	PP	LC/F			+													+			-
(231), 2005	2	Serum	PP	GC/MS			+													+			-
(232), 2005	2	Plasma	PP	LC/MS/MS	+						+												8 metabolites
(233), 2006	2	Serum	PP	LC/UV	+																	+	CBZ-E
(234), 2011	2	Plasma	PP	LC/MS/MS			+		+														-

CBZ-E: carbamazepine-10, 11 epoxide, CBZ-Diol: carbamazepine -10-11-Diol, HPPH: 5-(4-hydroxyphenyl)-5-phenylhydantoin, MHD: 10-hydroxycarbamazepine, FBM: felbamate, PRM: primidone, ESM: ethosuximide, P.P: protein precipitation, LLE: liquid-liquid extraction, FID: flame ionization detector, DAD: dual array detector, NPD: nitrogen phosphorus detector, MECC: micellar electrokinetic capillary chromatography, SPME: solid-phase microextraction, GC/TSD: gas chromatography with thermionic specific detection, CE: capillary electrophoresis, CED: coulometric electrochemical detection.

Finally, all of these studies were applied to investigate the pharmacokinetics of these drugs or to monitor their concentrations in the therapeutic regimens of epilepsy but no studies have been found for their potential misuse in DFC either as a single dose or multiple doses. In other words, all these methods analyzed the drugs within the therapeutic range in serum and plasma with some including overdose concentrations. No studies were found about the stability of the newer AEDs in postmortem human samples. Such studies are very important in forensic cases where a delay in obtaining and analyzing the samples is not unusual.

1.10 LC/MS/(MS) Role in Forensic Toxicology

As the compounds of interest are usually unknown, the first step before quantification should be the screening and identification of these compounds. Due to serious clinical or forensic consequences, high selectivity and reliable methods are required. Gas chromatography-mass spectrometry is still the most widely used method in all toxicological applications. Recently, liquid chromatography coupled with single or tandem mass spectrometry has become significantly more important in routine toxicological analysis. LC/MS is complementary to GC/MS, specifically for the quantification of thermo-labile, low-dose, high molecular mass and polar compounds (175).

The high sensitivity of LC/MS/(MS) appears to be a prerequisite in DFC especially when samples are collected many days after ingestion of the last therapeutic dose. It seems to be the best instrumentation to increase the likelihood of detection of drugs in biological samples (176). Furthermore, this technique needs less special conditions than other procedures such as immunoassay analysis. Immunoassay methods which are applied in the routine laboratory to screen for AEDs are very expensive and time consuming. Moreover, some AEDs (pregabalin, vigabatrin, tiagabine and lacosamide) have no immunoassay method available (198).

With respect to sample preparation, LC/MS/(MS) methods are more effective, productive and less time consuming compared to gas chromatography. GC/MS methods require a sample that is volatile or thermally stable with or without derivatization whereas LC/MS/(MS) has the advantage of being able to directly separate and analyze almost anything that is soluble in the mobile phase. This feature allows a wide range of drugs to be detected using LC/MS/(MS) since most drugs administered through ingestion, inhalation or injection are water soluble (235).

1.10.1 LC/MS/MS principle

LC/MS/(MS) is a high performance liquid chromatography instrument (HPLC) coupled with a tandem quadruple mass spectrometer. A mass spectrometer consists of two main components; the ion source which is responsible for ionizing the molecules and the mass analyser that sorts the ions according to their mass to charge ratios (m/z). There are different types of ion source and mass analyser that suit different classes of compounds.

1.10.1.1 Ion Sources

The primary focus on LC/MS research over the last decade has been to improve the separation of the analyte of interest from the mobile phase and enhance its ionization. The introduction of atmospheric pressure ionization (API) was a milestone in chromatography history because it greatly expanded the number of chemicals that could be analysed by LC/MS. Using API, the molecule is ionized first at atmospheric pressure before it is mechanically and electrostatically separated from the neutral solvent particles. The three most common API techniques are (236, 237) :

a) Electrospray Ionization (ESI):

This source generates analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed into a chamber at atmospheric pressure in the presence of a strong electrostatic field which causes further dissociation of the analyte molecules and is heated using a drying gas that evaporates the solvent in the droplets (Figure 1-20).

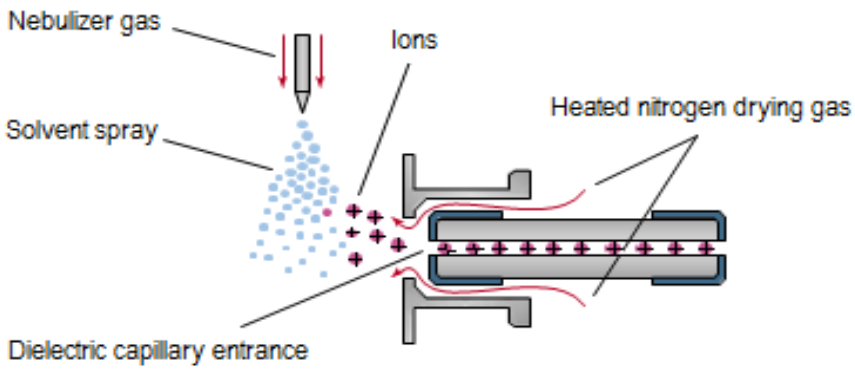


Figure 1-20: ESI Source.

"Reproduced with Permission From Agilent Technologies" (236).

As the droplets shrink, the charge concentration in the droplets increases. Eventually, the repulsive force between ions with like charges exceeds the cohesive forces and ions are desorbed into the gas phase. These ions pass through a capillary sampling orifice into the mass analyzer. Electrospray is especially useful for analyzing large and small molecules.

b) Atmospheric Pressure Chemical Ionization (APCI)

In this source, the LC eluent is sprayed through a heated vaporizer (250°C– 400°C) at atmospheric pressure which turns the liquid to gas phase. The resulting gas-phase solvent molecules are ionized by electrons discharged from a corona needle. The solvent ions then transfer charge to the analyte molecules through chemical ionization. The analyte ions pass through a capillary sampling orifice into the mass analyzer (Figure 1-21). APCI is applicable to a wide range of polar and nonpolar molecules; however, APCI is less well-suited than electrospray for analysis of thermally unstable large molecules because it involves high temperatures.

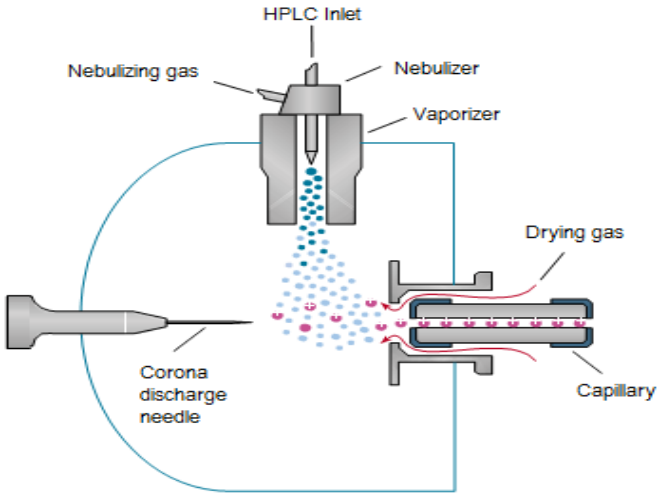


Figure 1-21: APCI Source.

"Reproduced with Permission From Agilent Technologies" (236).

c) Atmospheric Pressure Photo Ionization (APPI)

This is a relatively new technique. As in APCI, a vaporizer converts the LC eluent to the gas phase. A discharge lamp generates photons in a narrow range of ionization energies (Figure 1-22). The range of energies is carefully chosen to ionize as many analyte molecules as possible while minimizing the ionization of solvent molecules. The resulting ions pass through a capillary sampling orifice into the mass analyzer. APPI, similar to APCI is applicable to a wide range of polar and nonpolar molecules, however, it has also

showed promising results for highly nonpolar compounds when a very low flow rate was required (< 100µL/min) whereas APCI showed a low sensitivity.

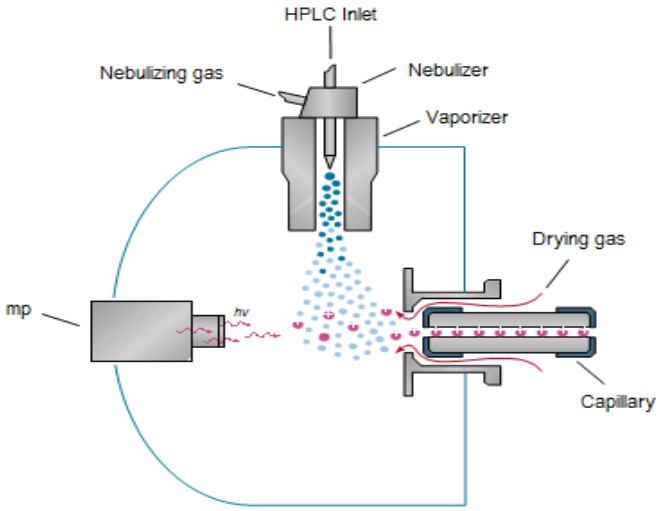


Figure 1-22: APPI Source.
"Reproduced with Permission From Agilent Technologies" (236).

1.10.1.2 Mass Analyzers

There are three types of mass analyzers that are used most often with LC/MS (236-241):

a) Tandem Quadrupole

Quadrupole Mass Analyzers were first described by Paul and Steinwegen from the University of Bonn in 1953. Quadrupole mass analyzers consist of four rods arranged in a square. The analyte ions are directed down the center of the square. Voltages applied to the rods generate electromagnetic fields. These fields determine which mass-to-charge ratio of ions can pass through the filter at a given time.

Quadrupoles lend themselves to being coupled to one another. Often incorrectly referred to as 'Triple Quadrupole' instruments, they are actually "Tandem Quadrupole" mass analyzers. The two quadrupole mass analyzers are referred to as Q1 and Q3, with the Q2 being a collision cell only (Figure 1-23). Ions are accelerated into the collision cell, filled with an inert gas (Argon, Nitrogen or Helium), with sufficient velocity that collisions with the gas molecules cause the analyte to break apart to form fragments. This process is called Collision Induced Dissociation (CID). The main advantage of these tandem quadruple mass analyzers is greater structural information and enhanced selectivity and sensitivity.

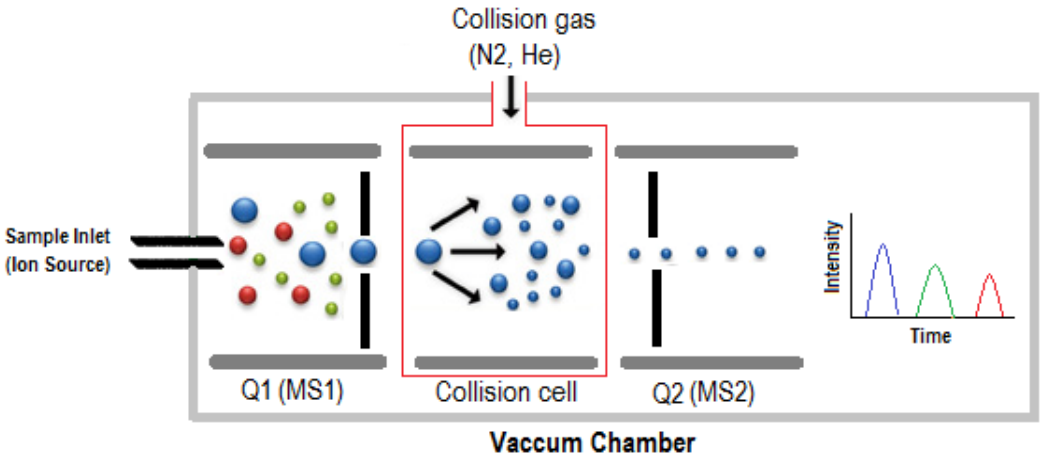


Figure 1-23: Tandem Quadrupole Mass Analyser.

b) Time-of-Flight

A uniform electromagnetic force is applied to all ions at the same time, causing them to accelerate down a flight tube. Lighter ions travel faster and arrive at the detector first, so the mass-to-charge ratios of the ions are determined by their arrival times (Figure 1-24). Time-of-flight mass analysers have a wide mass range and can be very accurate in their mass measurements.

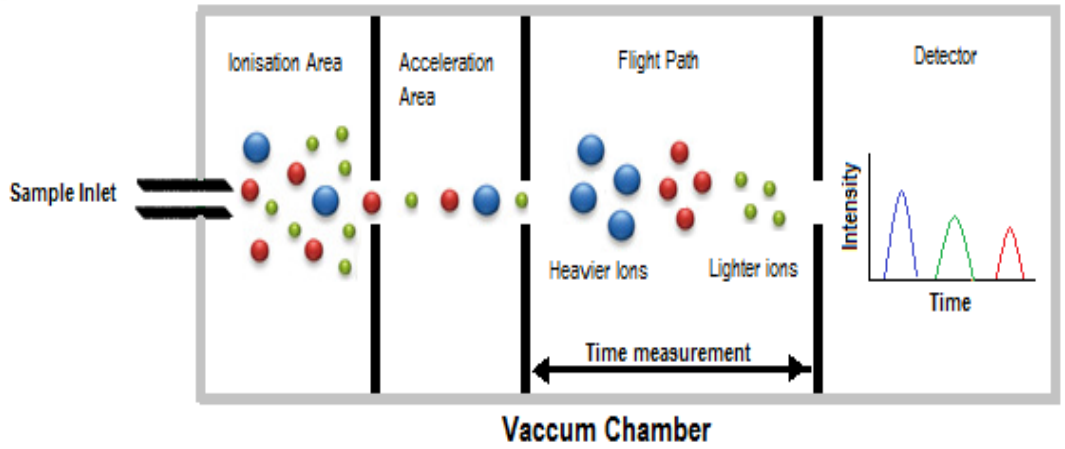


Figure 1-24: TOF Mass Analyser.

c) Ion Trap

An ion trap mass analyzer consists of a circular ring electrode plus two end caps that together form a chamber. Ions entering the chamber are “trapped” there by electromagnetic fields. Ion traps have the advantage of being able to perform multiple stages of mass spectrometry without additional mass analyzers (Figure 1-25).

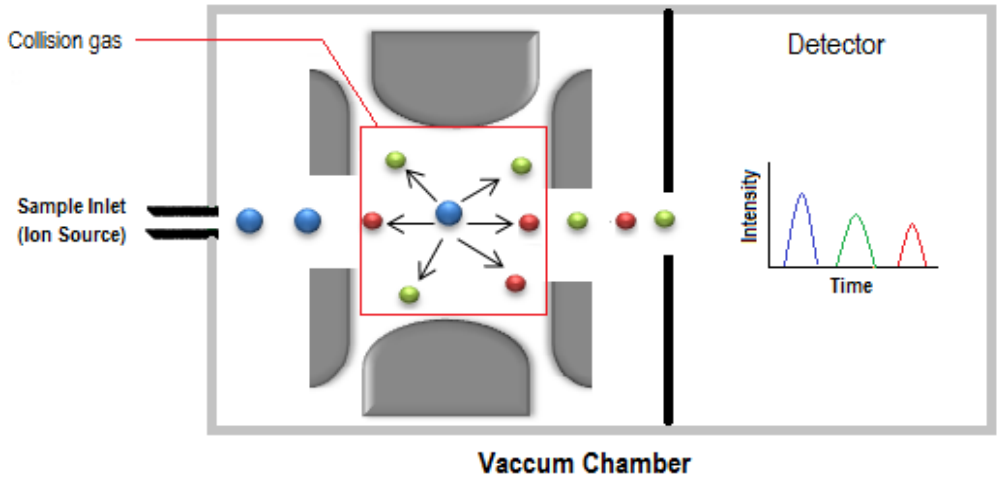


Figure 1-25: Ion Trap Analyser.

1.11 Conclusion

To sum up, the newer generation of antiepileptic drugs has clear sedative and depressive effects that allow them to be potential misused drugs in DFC. Some of them have already been abused (pregabalin, gabapentin) and awareness reports about their use have started to increase.

Numerous methods have been developed to analyze these drugs for TDM purposes in plasma and serum but few studies have been reported analyzing these drugs in post mortem blood, urine and hair. A wide range of analytical techniques either immunoassay or chromatographic analysis (GC and LC) with various kinds of detection applications (UV, DAD, FID…) have also been applied. The use of LC/MS/(MS) is considered as a prerequisite in forensic toxicology due to its ability to detect the low levels of drugs and their metabolites in the biological fluids at least six days after the ingestion of one therapeutic dose. This fact is very important in DFC cases where there is a delay in sample collection owing to late reporting of the offence by the victim. LC/MS/(MS) has been shown to be an ideal supplement to GC/MS/(MS). It may become the premium standard for quantification in clinical and forensic toxicology if the cost of the equipment can be reduced.

A number of simultaneous methods have been developed but none of these analyzed more than 11 drugs in a single run. Only one paper included 22 drugs but these drugs were divided into 3 groups with 3 different calibration curves depending on the abundance. None of these included lacosamide, eslicarbazepine acetate and retigabine.

Furthermore, all of these investigations were applied to study the pharmacokinetics of these drugs or to monitor their concentrations in the therapeutic regimens of epilepsy. Some of these included overdose concentrations as a result of studying the adverse effects of these drugs but few studies were found regarding concentrations of the newer AEDs in human postmortem blood or concentrations found in the days following the last dose where the concentration becomes very low. Such studies are very important in forensic cases where a delay in sampling is very common.

Hence, regarding the debate over these drugs and their ability to be misused in the press and scientific literature, this research highlights some of the AEDs which have concern and will evaluate their prevalence and abuse probability. Ultimately, a simultaneous analytical method was developed to analyze these drugs and their metabolites in human samples (postmortem blood, serum, plasma, urine and hair as an alternative sample) using liquid chromatography tandem mass spectrometry (LC/MS/MS). The method was validated according to the standard practices for method validation in forensic toxicology (SWGTOX, May 2013) and applied to authentic samples (242). It was used to study AED stability in blood, optimize the best extraction method with higher recovery to extract all AEDs in one analytical step in different matrices (protein precipitation, liquid-liquid extraction and solid phase extraction) and finally evaluate their prevalence and abuse potential among drug abusers and prisoners.

2 Method Development and Extraction Optimization of 17 AEDs in Whole Blood Using LC/MS/MS

2.1 Introduction

Various analytical tools have been developed for therapeutic drug monitoring (TDM) of AEDs (162, 163). Automated immunoassay methods have been the most widely used methods for the determination of anticonvulsants for TDM since the 1980s (183).

A number of simultaneous chromatographic assays for AEDs have been developed using GC (187), and HPLC coupled to an ultraviolet detection (190) or an evaporative light-scattering detector (194) in addition to fluorescence polarization immunoassay (183). The initial simultaneous assays, from the 1980s, concentrated on separating the older AEDs such as ethosuximide, primidone, phenytoin, phenobarbital, carbamazepine and carbamazepine-epoxide, with the inclusion or removal of one or more drug(s) or metabolite(s) such as lamotrigine, 5-para-hydroxyphenyl-5-phenylhydantoin (p-HPPH), ethyl-phenacemide, N-des-methyl-methsuximide and phenyl-2-ethyl-malonamide (178, 191, 196). However, these methods required time-consuming and difficult extraction procedures or relatively large sample volumes (about 1mL) as well as lengthy chromatographic run times that limited their throughput capacity and sensitivity (178). In addition, all methods which employ ultraviolet detection have the risk of interferences due to matrix, metabolites or other co-medications (197) and they are not applicable to some AEDs such as pregabalin, gabapentin, vigabatrin, topiramate and valproic acid due to a lack of a chromophore in their structures (199, 208).

Several methods have been described for the simultaneous LC/MS/(MS) analysis of a selection of AEDs (see 1.9.3). All of these methods were designed for TDM in plasma/serum over the therapeutic ranges. None of these methods were validated for postmortem blood over a wide concentration range including those associated with toxic levels.

The aim of this project was to develop a quantitive method for the analysis of the most commonly encountered AEDs in post mortem whole blood to include carbamazepine and its metabolite carbamazepine-10,11-epoxide, eslicarbazepine acetate, gabapentin,

lacosamide, lamotrigine, levetiracetam, oxcarbazepine, pregabalin, phenytoin and its metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin, retigabine (ezogabine in the US), topiramate, tiagabine, valproic acid, vigabatrin, and zonisamide, which would be suitable for routine forensic toxicological analysis.

2.2 Materials and Methods

2.2.1 Materials

Carbamazepine (CBZ), carbamazepine 10,11-epoxide (CBZO), gabapentin (GBP), lamotrigine (LTG) levetiracetam (LEV), oxcarbazepine (OXC), phenytoin (PHT), 5-(3-Hydroxyphenyl)-5-phenylhydantoin (p-HPPH), topiramate (TPR), vigabatrin (VIG), valproic acid (VPA) and zonisamide (ZNS) were purchased from Sigma Aldrich (Basingstoke, UK). Eslicarbazepine acetate (ESL), lacosamide (LAC), pregabalin (PGR), retigabine (RTG) and tiagabine (TIG) were obtained from LGC standards (Teddington, UK). All these drugs were purchased as powders.

Deuterated standards; gabapentin-D_{10} (GBP-D_{10}), pregabalin-D_6 (PGR-D_6) and topiramate-D_{12} (TPR-D_{12}), were obtained from LGC standards (Teddington, UK). Analog internal standards; 10, 11- dihydrocarbamazepine (CBZ-DiOH) and tolbutamide (TUB) were purchased from Sigma Aldrich (Basingstoke, UK).

Ammonium acetate and acetic acid were purchased from Sigma Aldrich (Basingstoke, UK). Acetonitrile (ACN), isopropanol (IPA) and methanol (MeOH) were supplied by VWR International Ltd (Lutterworth, UK). Double distilled water was obtained from the in-house Millipore® system.

2.2.2 Solutions Preparation

2.2.2.1 Preparation of Standard Solutions for Method Development

In order to optimize the fragmentor voltage and the collision energy required to analyse the drugs on LC/MS/MS, an individual stock solution for each drug was prepared at concentration of 1 g/L in methanol. These were prepared by adding 5 mg of the drug powder to a 5 mL volumetric flask and made up to volume with methanol. The stock solutions were further diluted 1:100 to obtain one working solution for each drug at 10 mg/L. These were achieved by adding 100 µL of the stock solution to a 10 mL volumetric

flask and made up to volume with mobile phase mixture (10 mM ammonium Acetate/ ACN: 50/50). These working solutions were used for method development.

2.2.2.2 Preparation of Calibrators and Quality Controls

AEDs were divided into 3 groups according to their proposed calibration curve ranges as explained in Table 2-1.

Table 2-1: AEDs Groups According to Their Proposed Calibration Curve Ranges.

Group 1 (0.5-50 mg/L)		Group 2 (0.05-10 mg/L)		Group 3 (5-300 mg/L)	
Drug	**Abbrev.**	**Drug**	**Abbrev.**	**Drug**	**Abbrev.**
Carbamazepine	CBZ	Oxcarbazepine	OXC	Levetiracetam	LEV
Carbamazepine epoxide	CBZO	Tiagabine	TIG	Vigabatrin	VIG
Eslicarbazepine acetate	ESL	Retigabine	RTG	Valproic acid	VPA
Gabapentin	GBP				
Lacosamide	LAC				
Lamotrigine	LTG				
Phenytoin	PHT				
5-(3-Hydroxyphenyl)-5-phenylhydantoin	p-HPPH				
Pregabalin	PGR				
Topiramate	TPR				
Zonisamide	ZNS				

Two separate stock solutions were prepared in methanol for each drug individually. One stock was used to prepare the calibration curve and the other was used to prepare the quality control samples (QCs). For LEV, VIG, VPA, CBZ and GBP, stocks were prepared at 10 g/L by dissolving 10 mg drug powder in 1 mL methanol. For all other drugs, stock solutions were prepared at 1 g/L by adding 5 mg drug powder to a 5 mL volumetric flask and made up to volume with methanol.

Three working solutions at concentrations of 100 mg/L for group 1 and 2 and 1 g/L for group 3 were prepared by combining certain volumes of the stock solutions and made up to volume with methanol as detailed in Table 2-2.

Eight calibration standard solutions were prepared in methanol by combining certain volumes of the three working solutions in 5 mL volumetric flasks and made up to volume with methanol to achieve the target concentrations as detailed in Table 2-3.

Table 2-2: Preparation of Calibrators and QCs Working Solutions.

Working Solution 1 (10 mL volumetric flask)			
Analyte	**Stock Concentration**	**Amount Added**	**Final Concentration**
CBZ	**10 g/L**	**100 µL**	100 mg/L
CBZO	1 g/L	1000 µL	100 mg/L
ESL	1 g/L	1000 µL	100 mg/L
GBP	**10 g/L**	**100 µL**	100 mg/L
LAC	1 g/L	1000 µL	100 mg/L
TPR	1 g/L	1000 µL	100 mg/L
LTG	1 g/L	1000 µL	100 mg/L
PGR	1 g/L	1000 µL	100 mg/L
PHT	1 g/L	1000 µL	100 mg/L
p-HPPH	1 g/L	1000 µL	100 mg/L
ZNS	1 g/L	1000 µL	100 mg/L
Working Solution 2 (10 mL volumetric flask)			
Analyte	**Stock Concentration**	**Amount Added**	**Final Concentration**
OXC	1 g/L	1000 µL	100 mg/L
TIG	1 g/L	1000 µL	100 mg/L
RTG	1 g/L	1000 µL	100 mg/L
Working Solution 3 (10 mL volumetric flask)			
Analyte	**Stock Concentration**	**Amount Added**	**Final Concentration**
LEV	10 g/L	1000 µL	1 g/L
VIG	10 g/L	1000 µL	1 g/L
VPA	10 g/L	1000 µL	1 g/L

Table 2-3: Preparation of the Calibration Curve Standards.

Volumetric Flask (5 mL) No.	Calibrator No.	Amount Added (µL)			Final Concentration (mg/L)		
		Working Solution 1	Working Solution 2	Working Solution 3	Group 1	Group 2	Group 3
1	Cal 1	25	2.5	25	0.5	0.05	5
2	Cal 2	50	5	50	1	0.1	10
3	Cal 3	125	12.5	125	2.5	0.25	25
4	Cal 4	250	25	250	5	0.5	50
5	Cal 5	500	50	375	10	1	75
6	Cal 6	1000	125	500	20	2.5	100
7	Cal 7	1750	250	1000	35	5	200
8	Cal 8	2500	500	1500	50	10	300

Three QC samples (low, medium and high) were directly made in whole blood. Using the second set of stock solutions, three working solutions were prepared as detailed earlier in Table 2-2. Three QCs were prepared in whole blood by combining certain volumes of the three working solutions in 10 mL volumetric flasks. Then, the methanol solvent was evaporated under nitrogen at 25ºC to avoid blood precipitation. The residue was finally

reconstituted with 10 mL whole blood to achieve the target concentrations as detailed in Table 2-4. QC concentrations were 3, 20 and 40 mg/L for group 1; 1, 4 and 8 mg/L for group 2; and 20, 120 and 200 mg/L for group 3. For each QC, 0.5 mL aliquots were placed into labelled 1.5 mL polystyrene screw cap tubes. All QCs and stock solutions were stored at -20°C, and working solutions were stored at 4°C.

Table 2-4: Preparation of QCs in Whole Blood.

Volumetric Flask (10 mL) No.	QC No.	Amount Added (µL)			Final Concentration (mg/L)		
		Working Solution 1	Working Solution 2	Working Solution 3	Group 1	Group 2	Group 3
1	QC 1	300	100	200	3	1	20
2	QC 2	2000	400	1250	20	4	120
3	QC 3	4000	800	2250	40	8	200

2.2.2.3 Preparation of Internal Standards

Five internal standards, GBP-D_{10}, PGR-D_6, TPR-D_{12}, TUB and CBZ-DiOH were used. GBP-D_{10}, PGR-D_6, TPR-D_{12} were readily purchased in methanol at concentration of 100 mg/L. TUB and CBZ-DiOH were purchased as a powder. An amount of 1 mg was dissolved in 10 mL methanol to obtain a 100 mg/L solution of TUB and CBZ-DiOH. Using a 100 mL volumetric flask, a combined internal standard solution was prepared at 10 mg/L and made up to volume with methanol as outlined in Table 2-5 below.

Table 2-5: Internal Standard Solution Preparation.

Internal Standard	Abbrev.	Stock Solution (mg/L)	Amount Added (mL)	Final Concentration (mg/L)
Gabapentin-D_{10}	GBP-D_{10}	100	1	10
Pregabalin-D_6	PGR-D_6	100	1	10
Topiramate-D_{12}	TPR-D_{12}	100	1	10
Tolbutamide	TUB	100	1	10
10,11 Dihydro carbamazepine	CBZ-DiOH	100	1	10

2.2.2.4 Preparation of Blank Blood

Whole blood was prepared using packed red cell pouches which were obtained from the Blood Bank at the Western Infirmary Hospital (Glasgow, UK) and were frozen on receipt

within Forensic Medicine and Science (FMS). The packed red blood cells was fully defrosted and then measured and diluted 1:1 with 1% saline solution. Saline solution was prepared by adding 9.5g sodium chloride into a 1L volumetric flask and dissolving it in deionised water by making up to the mark. The blank whole blood was used to prepare quality control samples.

2.2.2.5 Preparation of Formic Acid 0.1% (pH=2.8)

1 mL of concentrated formic acid was placed in a 1L volumetric flask and made up to volume with deionised water.

2.2.2.6 Preparation of 10 mM Ammonium Formate (pH =3)

Ammonium formate (0.631g) was added to a 1L volumetric flask and made up to volume with deionised water.

2.2.2.7 Preparation of 10 mM Ammonium Acetate (pH = 5)

Ammonium acetate (0.77g) and 200 µL of concentrated acetic acid were added to a 1L volumetric flask and made up to volume with deionised water.

2.2.2.8 Preparation of 10 mM Ammonium Carbonate (pH = 9.3)

Ammonium carbonate (0.785g) was added to a 500 mL volumetric flask and made up to volume with deionised water.

2.2.2.9 Preparation of 2 M Ammonium Acetate

Ammonium acetate (15.42 g) was added to a 100 mL volumetric flask and made up to volume with deionised water.

2.2.2.10 Preparation of 2 mM Ammonium Acetate in Methanol

A 1 mL aliquot of 2 M ammonium acetate was added to a 1L volumetric flask and made up to volume with methanol.

2.2.2.11 Preparation of 2 mM Ammonium Acetate (pH = 6.8)

A 1 mL aliquot of 2 M ammonium acetate was added to a 1L volumetric flask and made up to volume with deionised water.

2.2.2.12 Preparation of Wash Solution

Wash solution was prepared using 25% isopropanol, 25% ACN, 25% MeOH and 25% 2mM ammonium acetate in 0.1% formic acid.

2.2.3 Instrumentation

An Agilent LC/MS/MS triple quadruple G6420A mass spectrometer equipped with an electrospray ionization (ESI) source, Agilent 1200 Series Auto sampler SL, Agilent 1200 Series Binary Pump SL with degasser and Agilent 1200 Series Thermostatted Column Compartment SL was used. The turbo ion-spray interface was operated in both positive- and negative-ion modes with nitrogen as the collision gas. The Agilent Mass-Hunter Workstation software (version: B.01.05) was used for system control and data acquisition. Optimizer software was used to optimize the product ions and their fragmentor voltages and collision energies.

2.2.4 Optimisation of the Fragmentor Voltage and Collision Energy

An evaluation of the different tuning techniques available was carried out in order to determine the mass spectrometry characterization of the 15 antiepileptic drugs and 2 of their metabolites. All compounds of interest and internal standards were individually tuned using sample injection program and Optimizer software to detect the precursor ions and optimize their product ions, fragmentor voltage and collision energy.

Optimizer is an automated MS method development Software installed with Agilent Mass-Hunter Acquisition software. It automatically optimizes the data acquisition parameters for multiple-reaction monitoring mode (MRM) for each compound analysed. Specifically, it automates the selection of the best precursor ions and their fragmentor voltage, the selection of the best product ions and the collision energy values for each transition. A mixture of 17 AEDs and 5 internal standards was dissolved in mobile phase and transferred to a LC vial. 20 µL was injected through the autosampler. At the end of the analysis, the software generated a report with all required details (see Appendix 2-1).

For the sample injection program, analytes were prepared individually in the mobile phase at concentration of 10 mg/L and a 20 µL volume was injected through the autosampler. The tuning was carried out in 4 steps for each analyte as follows:

Step 1: MS1 scan method to determine the precursor ion of each analyte.

Step 2: Fragmentor voltage optimization for each precursor ion at 20, 50, 80, 110, 140, 170 and 240 eV. This was achieved by building up 7 individual methods with different fragmentor values.

Step 3: Product ion mode method to determine the product ions profile using the results from step 1 and 2.

Step 4: Optimize the collision energy of each transition over a range between 0 and 40 V by building up different methods and increasing the collision energy value by 5 each time.

The results of this method were saved as data files and processed using Mass-Hunter Qualitative program by overlapping analyte chromatograms to compare their peaks height and area.

LC/MS/MS operation conditions and mobile phase gradient used at this stage are detailed in Table 2-6 and Figure 2-1.

Table 2-6: Summary of LC and Ion Source Parameters Used During Method Development.

LC Parameters	
Column	Gemini C18 column (150 x 2.1 mm, 5 µm) with guard column of the same packing material
Mobile phase	10 mM ammonium acetate in water/ACN (80:20).
Column temperature	25 ºC
Flow rate	300 µL/min
Mass Spectrometry Parameters	
Operating mode	ESI-in positive and negative mode
Gas temperature	300 ºC
Gas flow	11 L/min
Nebulizer pressure	15 PSI
Capillary voltage	4000 V

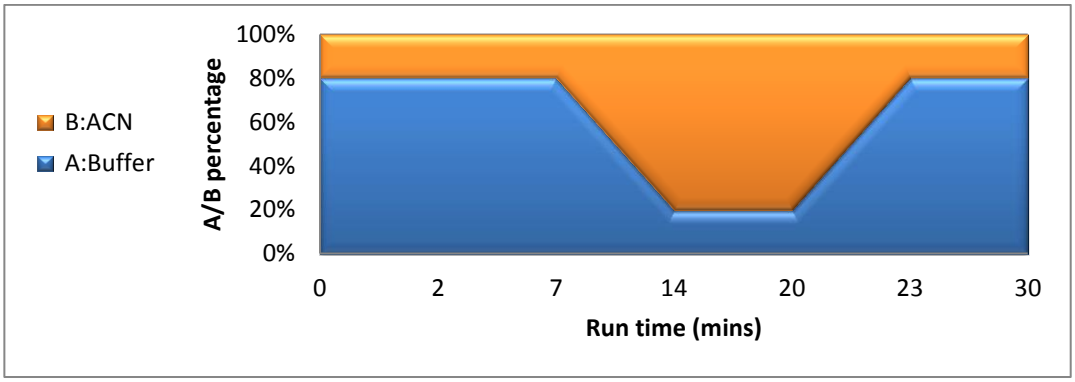


Figure 2-1: Mobile Phase Gradient System Applied at Method Development Stage.

2.2.5 Optimisation of Nebulizer Gas Pressure

The nebulizer gas (N_2 in this study) pressure (Neb) is an essential component to disperse the emerging solution of the sample into small droplets and to direct the droplets on the trajectory chosen for optimal sampling. Nebulizer gas pressure was optimized at 15, 25 and 35 PSI in order to obtain the highest sensitivity.

2.2.6 Optimization of Mobile Phase

A suitable eluent for electrospray ionisation should contain an organic modifier (methanol or acetonitrile) and a volatile buffer, the concentration of which can be critical; concentrations that are too high may result in the suppression of the analyte signal, while concentrations that are too low may lead to poor peak shape and efficiency. In order to determine the most suitable mobile phase composition for AEDs of interest, an investigation into its aqueous and organic components was carried out.

2.2.6.1 Aqueous Phase Additives

When developing a robust method, it is desirable to select a mobile phase with a final pH that is at least two units away from the pKa. However, this rule was a bit challenging to apply due to the wide range of AEDs of interest. This experiment was carried out for each drug separately to investigate the effect of various buffers on the AEDs with the aim of choosing the buffer which produced good chromatograms and high abundances for all the analytes.

Four buffers with different additives were tested to achieve the highest response of the drugs under the same operating conditions (Table 2-7). Four separate non-extracted standards of each analyte were prepared using 200 µL aliquot of the 10 mg/L working

solution, dried under nitrogen. Each sample was reconstituted with 100 µL of one selected mobile phase (buffer/organic, 80:20). A 10 µL volume was injected in triplicate for each mobile phase. This experiment was not carried out for CBZO, CBZ-DiOH, p-HPPH, RTG and ESL which were added to the method at a later stage.

Table 2-7: A List of Buffers Tested for Mobile Phase Optimisation.

pH	Buffer	Organic phase	MP percentages
2.8	0.1% Formic acid	Acetonitrile	(80:20)
3.0	10 mM Ammonium formate	Acetonitrile	(80:20)
5.0	10 mM Ammonium acetate	Acetonitrile	(80:20)
9.3	10 mM Ammonium carbonate	Acetonitrile	(80:20)

2.2.6.2 Mobile Phase pH

Volatile, low molecular weight organic acids (eg. formic and acetic acid) are commonly used as additives in LC/MS to improve ionization and resolution. The addition of acid was evaluated by adding glacial acetic acid to the mobile phase of choice, 10 mM ammonium acetate (pH= 6.8), to achieve a pH of 4.0, 4.5 and 5.5. Non-extracted standards of each analyte were prepared in duplicate using 200 µL aliquot of the 10 mg/L working solution, dried under nitrogen. Each sample was reconstituted with 100µL of one selected mobile phase (buffer/organic, 80:20). Acetonitrile was used for the organic phase. A 10 µL volume was injected in triplicate for each mobile phase. The average peak area was calculated for each analyte.

2.2.6.3 Aqueous Phase Molarity

The mobile phase additive concentration (molarity) has a significant effect on the analyte electro-spray response. It has been reported that increasing the additive concentration leads to a dramatic decrease in analyte sensitivity due to its suppressive effect on electro spray ionization (243). In order to investigate the effect of the mobile phase additive on AEDs ionization, two mobile phases were compared using low and high molarity; 2 and 10 mM ammonium acetate. Non-extracted samples were prepared and injected as before in 2.2.6.2.

2.2.6.4 Organic Phase

Substitution of methanol for acetonitrile in the HPLC mobile phase has been shown to produce a significant difference in the electrospray ionization of a variety of compounds.

(244). A comparison was carried out between methanol and acetonitrile to investigate their effects on the chromatograms of the analytes, their ion abundances and peak shape.

2.2.7 Optimization of LC Column Stationary Phase and Temperature

AEDs of interest are a group of acidic, basic and amphoteric compounds with a wide range of pKa and different polarities; hence, a simple and general column which can tolerate a wide range of pH was required in order to elute all the compounds. A typical C18 column was used as a starting point (Table 2-6). In order to achieve better separation and cleaner chromatograms, three different columns were compared with the previous Gemini C18 column (Table 2-8). Agilent C18 XDB column and Agilent C8 XDB column were used to compare the effect of column dimensions on resolution and analyte elution. Obelisc R column has reversed phase (C18) characteristics in addition to the presence of ionic groups and a long hydrophobic chain which offer multiple separation modes (ion exchange, reverse and normal phase). The column offers additional retention and different mechanisms of reaction that should help to resolve the target compounds depending on their structure and chemical groups.

Table 2-8: Chromatographic Column Properties.

Column	Length (mm)	Internal Diameter (mm)	Particle Size (µm)
Gemini C18 Pheomenex	150	2.0	5
Agilent C18 XDB	50	4.6	1.8
Agilent C8 XDB	150	4.6	5
Obelisc R (mixed mode)	150	4.6	5

After choosing the best mobile phase and column system for analysing all AEDs simultaneously, the column temperature was optimized at 25, 40 and 50°C.

2.2.8 Optimization of Chromatographic Separation

A separation issue appeared during the method development stage for 4 of the drugs/metabolites. These drugs are carbamazepine and eslicarbazepine acetate, oxcarbazepine and carbamazepine 10, 11-epoxide, which are all derivatives of carbamazepine having similar structures. In order to separate these drugs and get a good resolution, several experiments were carried out by changing the mobile phase gradient system and the flow rate.

The flow rate was changed using three different rates; 0.1, 0.2 and 0.3 mL/min. Different gradient systems were applied by modifying the aqueous/organic phase percentage of the mobile phase in order to achieve the best separation for all the compounds in one analysis as illustrated in Table 2-9.

Table 2-9: Mobile Phase Gradient Systems Experiments in Order to Achieve the Required Chromatographic Separation.

Time (mins)	System 1		System 2		System 3		System 4		System 5	
	A%*	B%*	A%	B%	A%	B%	A%	B%	A%	B%
0	80	20	80	20	80	20	90	10	90	10
2	80	20	60	40	50	50	90	10	50	50
10	10	90	40	60	50	50	10	90	50	50
10.5	10	90	10	90	10	90	10	90	10	90
11.5	10	90	10	90	10	90	10	90	10	90
12	80	20	80	20	80	20	90	10	90	10
20	80	20	80	20	80	20	90	10	90	10

*A: 10 mM ammonium acetate, B: ACN.

2.2.9 Optimization of LC/MS/MS Operation Mode, MRM vs DMRM

Presently, many reports on the quantitative analysis of AEDs using LC/MS/MS are based on multiple reaction monitoring (MRM), which has been widely applied to biomarker validation and other target quantification because of its well-known selectivity and sensitivity. This method, however, has critical limitations especially when targeting drugs with very low LOD and LOQ. Since transitions are monitored in the whole time period of a standard MRM time segment, the rate of false positive detection (i.e. peak misidentification) increases. With improved sensitivity and better LOD and LOQ, dynamic multiple reaction monitoring (DMRM) could offer a good solution to this issue. This technique monitors the analytes only around the expected retention time, and thus decreases the number of concurrent MRM transitions, allowing both the cycle time and the dwell time to be automatically optimized for the highest sensitivity, accuracy and reproducibility which in turn decrease limit of detection and quantification. Moreover, DMRM allows the monitoring of more MRM transitions in a single run without compromising data quality. Hence, it was decided to apply DMRM mode for MS analysis of AEDs (245).

2.2.10 Extraction Optimization - Solid Phase Extraction vs Protein Precipitation

Before introducing any samples to LC/MS/MS, the analytes first need to be extracted from the matrix and concentrated. The method needs to be able to wash enough interfering compounds away without diminishing the recovery of the analytes.

Following development of the instrument method, extraction experiments were carried out to optimize the best conditions to extract the 17 AEDs as a mixture with highest recovery and minimum matrix effect. Additionally, peak shape, reproducibility, time of extraction, linearity range and the cleanliness of the extracts were evaluated when optimizing extraction methods.

It was decided to evaluate two extraction methods modified from the literature; solid phase extraction (SPE) using Strata-X 33-mg (Phenomenex) cartridges (30 mg/1mL) and protein precipitation.

a) Solid Phase Extraction: Strata-X 33-mg (Phenomenex) cartridges are made of reversed phase polymeric sorbent that gives strong retention of neutral, acidic, or basic compounds (246). This sorbent relies on 3 mechanisms of retention: pi-pi bonding, hydrogen bonding (dipole-dipole interactions) and hydrophobic interaction.

A volume of 100µL spiked blood was diluted with 400 µL 0.1% formic acid/MeOH (80:20) mixture. The cartridges were conditioned with 1 mL MeOH then equilibrated with 1 mL deionised water. After loading the diluted sample, it was allowed to drip through with no vacuum applied, then the cartridges were washed with 1 mL 20% MeOH and left to dry with full vacuum applied for 2 minutes. Elution was achieved using 1 mL MeOH. A 100 µL internal standards mix solution (TUB, CBZ-DiOH and GBP-D_{10}) at a concentration of 10 mg/L was added to the eluted sample which was then evaporated under a stream of nitrogen at 25 °C, reconstituted with 200 µL mobile phase (80/20: A/B) and transferred to a LC vial. A 10 µL volume was injected into the LC/MS/MS.

b) Protein Precipitation with MeOH: A 100 µL aliquot of blood was transferred to a 2-mL snap top polypropylene micro-centrifuge tube followed by 100 µL of mixed working standard solution at 10 mg/L and 300 µL of MeOH. This was vortex mixed for 10 seconds and centrifuged for 10 minutes at 5000 rpm. A 100 µL internal standard mix solution (TUB, CBZ-DiOH and GBP-D_{10}) at a concentration of 10 mg/L was added to the

supernatant which was then evaporated under a nitrogen stream at 25 °C, reconstituted with 200 µL mobile phase (80/20: A/B) and transferred to a LC vial. A 10 µL volume was injected into LC/MS/MS.

For both extractions, standards were prepared in triplicate and injected in duplicate at a concentration of 10 mg/L. In order to calculate the recovery, a non-extracted standard at the same concentration was also prepared at the same time in triplicate. Internal standards mix solution was added after the extraction to all extracted and non-extracted standards in order to eliminate the matrix effect on the internal standard and evaluate the absolute recovery of the analytes. The peak area ratios for the analyte and its respective internal standard were calculated. The absolute recovery was determined for each analyte by dividing the extracted STD/IS ratio by that of the non-extracted STD/IS ratio at the same concentration and multiplying by 100.

2.2.11 Sample Reconstitution Optimization

Due to the different chemical structures of the AEDs of interest, the analytes exhibited different intensities which required different reconstitution volumes in order to attain good linearity. Hence, the effect of the reconstitution solution composition and its volume on linearity and lower limit of quantification had been optimized.

The use of different reconstitution volumes for the same extracted sample in order to get the desirable results was previously reported in the literature. A method published recently had to divide the AEDs into 3 groups with 3 different dilution volumes in order to achieve good sensitivity and acceptable linearity (203). In other words, three different sets of calibrations had to be prepared which increased the time spent on the analysis and increased the cost on consumables.

In order to achieve one dilution step for all the AEDs with good linearity, the following parameters were investigated; sample reconstitution solution volume and reconstitution solution composition effect on drug solubility.

2.2.11.1 Sample Reconstitution Volume

A series of dilution volumes were evaluated starting at 1.0, 2.0, 3.0 and 4.0 mL. The mobile phase (80/20: A/B) was used as a reconstitution solution at this stage.

Samples were prepared in triplicate as follows; a 100µL aliquot of blood was transferred to a 2-mL micro-centrifuge tube, 100 µL of standard mix solution at 10 mg/L, 100 µL of internal standard and 200 µL MeOH were added, vortex mixed for 10 seconds and centrifuged for 10 minutes at 5000 rpm. The supernatant was then evaporated under a nitrogen stream at 25°C and reconstituted with mobile phase (80/20: A/B) to achieve the volumes mentioned earlier. 1 mL was transferred to a LC vial and 10 µL injected into the LC/MS/MS. The lower limit of detection and linearity for each analyte were compared.

2.2.11.2 AED Solubility in Reconstitution Solution

Seven different reconstitution solution mixtures including the mobile phase were evaluated. Non-extracted standards were used for this experiment in order to eliminate the effect of the matrix on the drug responses. Non-extracted analytes were spiked directly into the reconstitution solutions to obtain a final concentration of 2, 0.2 and 20 mg/L for group 1, 2 and 3 respectively as detailed in Table 2-10 (See Table 2-2 for working solution preparation). A volume of 1 mL was transferred to a LC vial and 10 µL was injected. Analyte response and peak shape were compared by overlapping the chromatograms of each analyte and the highest abundance was recorded.

Table 2-10: Preparation of AEDs Samples to Evaluate Their Solubility in Different Reconstitution Solutions

Amount Added (µL) to 10 mL Volumetric Flask			Reconstitution Solution Volume (µL)	Final Concentration (mg/L)		
Working Solution 1 (100 mg/L)	Working Solution 2 (100 mg/L)	Working Solution 3 (1 g/L)		Group 1	Group 2	Group 3
200	20	200	9580	2	0.2	20

The following reconstitution solution mixtures were used:

1. 2 mM ammonium acetate/MeOH (80:20)
2. 10% MeOH in water
3. 10% MeOH/0.1% formic acid in water
4. 5% ACN in water
5. 5% ACN/0.1% formic acid in water
6. 0.1% formic acid
7. Water

2.2.12 Investigation into Protein Precipitation Extraction Conditions

The aim of this study was to optimize the effect of sample preparation steps on the protein precipitation extraction of 15 AEDs and 2 metabolites to create an efficient routine forensic toxicological method. Steps evaluated in the study are the solvents used for extraction, the centrifuge duration and speed and finally the effect of blood haemolysis with water before extraction on recovery.

For this study two standards were prepared; non-extracted and spiked whole blood at the same concentrations; 10 mg/L for group 1, 2.5 mg/L for group 2 and 100 mg/L for group 3, as outlined in Table 2-11.

Table 2-11: Preparation of AEDs Standards in Whole Blood for Extraction Optimization Experiments.

AEDs Standard	Amount Added to 10 mL Volumetric Flask (µL)			Whole Blood (µL)	MeOH (µL)	Final Concentration (mg/L)		
	Working Solution 1	Working Solution 2	Working Solution 3			Group 1	Group 2	Group 3
Whole blood Standard	1000	25	100	8875	-	10	2.5	10
Non-extracted standard	1000	25	100	-	8875	10	2.5	10

Two internal standards were used at concentration of 10 mg/L; 10, 11 dihydro-carbamazepine (CBZ-DiOH) for CBZ, CBZO, ESL and OXC and tulbotamide for the other drugs (See Table 2-2 and Table 2-5 for working and internal standards solutions preparation).

2.2.12.1 Effects of Extraction Solvent

The effects of seven different solvent mixtures were evaluated for the protein precipitation extraction of 17 AEDs.

Seven sets; 3 samples each, of spiked whole blood samples were prepared. Each set was extracted with one of the 7 solvent mixtures shown in Table 2-12 as follows; 100 µL of spiked blood was transferred to a 2-mL micro-centrifuge tube, 400 µL of solvent mixture were added, vortex mixed for 10 seconds and centrifuged for 10 minutes at 5000 rpm. A 200 µL aliquot of the supernatant was transferred to a LC vial, spiked with 20 µL of

internal standard (10 mg/L) and diluted to 1.5 mL with deionized water. 10 µL were injected into the LC/MS/MS.

In order to calculate the recovery, seven sets of non-extracted samples at the same concentration (see Table 2-11) were also prepared at the same time in triplicate as following; 100 µL of the standard solution was diluted with 400 µL of one solvent mixture. A 200 µL aliquot of the diluted standard was transferred to a LC vial, spiked with 20 µL of internal standard (10 mg/L) and diluted to 1.5 mL with deionized water.

Internal standard was added after the extraction to all extracted and non-extracted samples. The ratios between the analyte mean peak area and its respective deuterated standard were calculated. The absolute recovery was determined for each analyte by dividing the extracted STD/IS ratio by that of the non-extracted STD/IS ratio at the same concentration and multiplying by 100.

Table 2-12: Solvent Combinations Tested.

Set No.	*Solvent Mixture*	*Total Volume (µL)*
Set 1	Methanol	400
Set 2	Ethanol	400
Set 3	Acetonitrile	400
Set 4	Acetone	400
Set 5	MeOH/ACN/EtOH	200:100:100
Set 6	MeOH/ACN/Acetone	200:100:100
Set 7	MeOH/EtOH/Acetone	200:100:100

2.2.12.2 Effect of Centrifuge Speed and Duration

The effect of centrifuge duration on extraction yield was evaluated at 10, 15 and 20 minutes and its speed was assessed at 3000, 5000 and 10000 rpm.

Six sets of spiked whole blood samples were prepared in triplicate. Three sets were centrifuged at 5000 rpm for 10, 15 and 20 minutes, and the other three sets were centrifuged for 10 minutes at 3000, 5000 and 10000 rpm. All sets were extracted as in 2.2.12.1. One set of non-extracted standards at the same concentration was also prepared

at the same time in triplicate and absolute recovery was calculated as mentioned earlier in 2.2.12.1.

2.2.12.3 Effects of Water Haemolysis

Water haemolysis of blood was reported to improve the extraction yield of some drugs by uniformly dissolving blood components and providing a desirable consistency after vortex-mixing (247). In order to assess the effect of water haemolysis on AEDs extraction, a set of 3 spiked whole blood samples were extracted with MeOH. Before the extraction, 100 µL deionized water was added to 100 µL of spiked blood (1:1 v/v) and vortex mixed for 10 seconds. A 100 µL of haemolysed blood was extracted with 400 µL MeOH and centrifuged for 10 minutes at 5000 rpm. Three non-extracted samples were prepared at the same time. Both sets were spiked with 20 µL internal standard after extraction. Recovery was calculated as mentioned earlier in 2.2.12.1.

2.2.12.4 Matrix Effect Evaluation

Finally, the matrix effect was evaluated for two selected extraction methods (methanol and acetonitrile) using the post-extraction addition approach to assess ionization suppression/enhancement.

Recovery and matrix effect in this experiment were assessed using the Matuszewski strategy (248). Three sets of QCs at a concentration of 10 mg/L for group 1 and 3 and 2.5 mg/L for group 2 (Table 2-11) were prepared as follows:

Set 1 (Non-extracted QCs): 6 QCs were prepared using non-extracted standards and internal standards. A 100 µL aliquot of the QC solution with 100 µL of the internal standard solution was added to a micro centrifuge tube. A 200 µL aliquot of methanol was added (total volume 400 µL) and mixed, then 200 µL of the sample was transferred to a LC vial and diluted to 1.5 mL with water.

Set 2 (Post extraction QCs): Blank blood was extracted as mentioned in 2.2.12.1 using 6 different sources (donors) of blood. QC solutions and internal standards were added *after* extraction.

Set 3 (Pre extraction QCs): Blank blood was spiked with QC solutions and internal standards *before* extraction and extracted as mentioned in 2.2.12.1 using the same 6 sources of blood.

After analysing the samples, the peak area was used to calculate the recovery, matrix effect and process efficiency using the following equation:

$$Recovery\ RE\ (\%) = Pre\ (3) / Post\ (2) \times 100$$

$$Process\ Efficiency\ PE\ (\%) = Pre\ (C) / Non\text{-}extracted\ (A) \times 100$$

$$Matrix\ Factor\ (MF) = Post\ (2) / Non\text{-}extracted\ (1)$$

MF is acceptable if the value is within 1±0.25.

If MF = 1, there is no matrix effects.

If MF <1, there is an ionisation suppression effect.

If MF >1, there is ionisation enhancement and/or analyte loss in the absence of matrix.

Finally, recoveries using both approaches, peak area/IS ratio and Matuszewski strategy were compared.

2.3 Results and Discussion

2.3.1 Optimisation of the Fragmentor Voltage and Collision Energy

Optimizer Software was used initially to investigate AEDs precursor and product ions, fragmentor voltage, collision energy and the ionization mode.

Using the automated software saved time and effort required for method development. However, the program did not predict the parameters needed for most AEDs of interest. For instance, in the case of CBZ and its derivatives; CBZO and OXC and their internal standard CBZ-DiOH, the program did not give any results in either negative or positive mode. On the other hand, Optimizer predicted a fragmentation pattern for p-HPPH in positive mode (Appendix 2-1) but the abundance was very low. This occurred also with

CBZO but in negative mode. As a result, the values were not sufficiently reliable to be used as a reference to build the acquisition method.

The sample injection program was used as an alternative procedure to tune the compounds of interest. All the analytes investigated in this study generated the prominent protonated molecular ion ([M+H]+) in positive-ion mode and the deprotonated molecular ion ([M-H]−) in negative ion mode. Based on signal intensity and fragmentation pattern, the analytes were divided into two ionization groups; positive ionisation mode including all AEDs except PHT, p-HPPH, TPR, TPR-D_{12}, TUB, VPA and ZNS which showed a negative ionisation mode.

The fragmentor voltage was in the range of 50–140 V for all AEDs and their internal standards. The optimum abundance of the precursor ions is illustrated in Figure 2-2. Collision energy was optimized to get the most abundant product ions using nitrogen gas for collision. Most analytes had optimum collision energy of less than 20 eV as illustrated in Figure 2-3. Although a qualitative ion and a quantitive ion is required for LC/MS/MS quantitive analysis, however, no fragment ions were observed in MS/MS spectra for valproic acid, so quantitative analysis was applied using the non-reactive transition m/z 143 for this drug and its retention time. These were the only criteria used to confirm valproic acid analysis. Table 2-13 summarizes the optimization data.

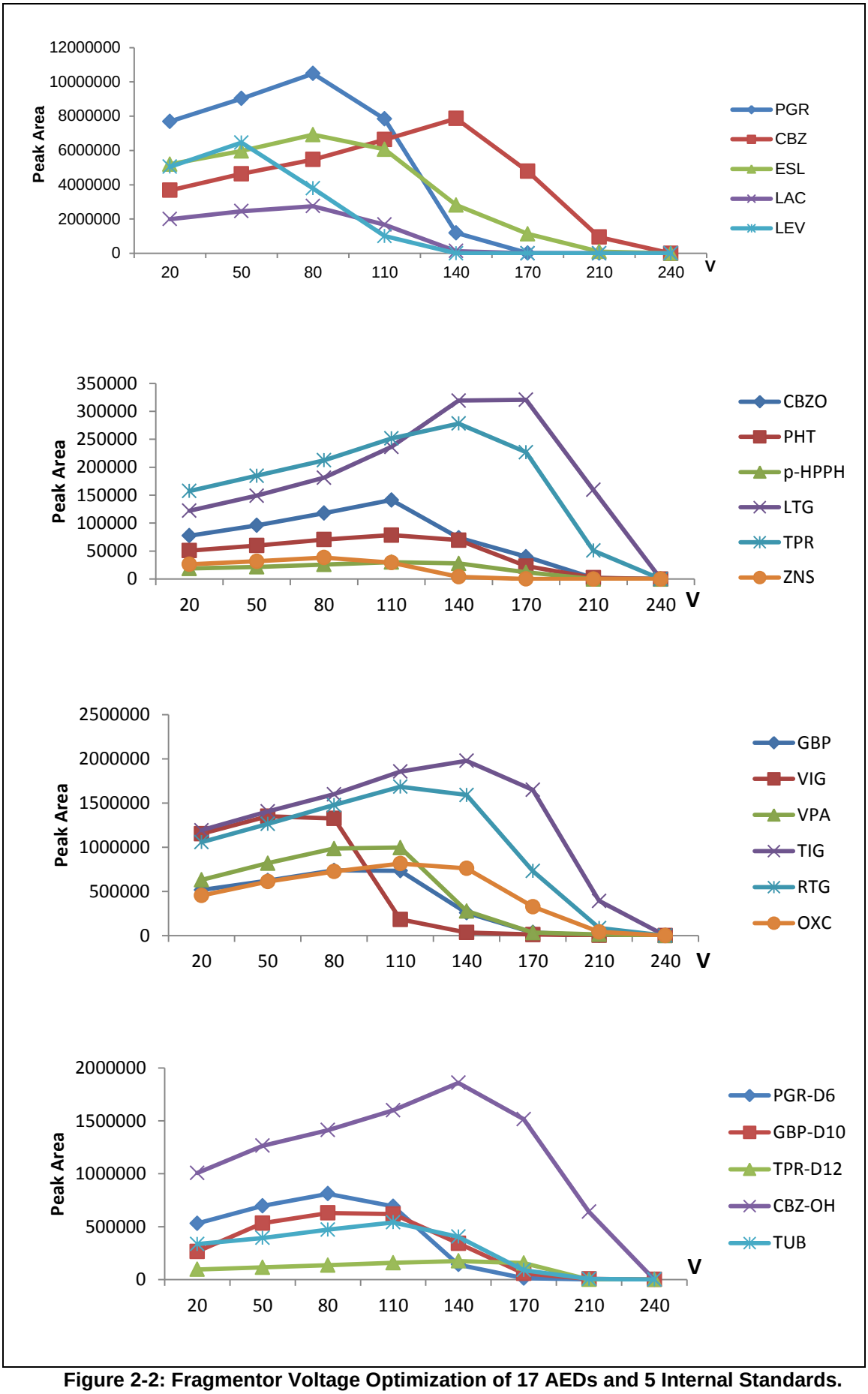


Figure 2-2: Fragmentor Voltage Optimization of 17 AEDs and 5 Internal Standards.

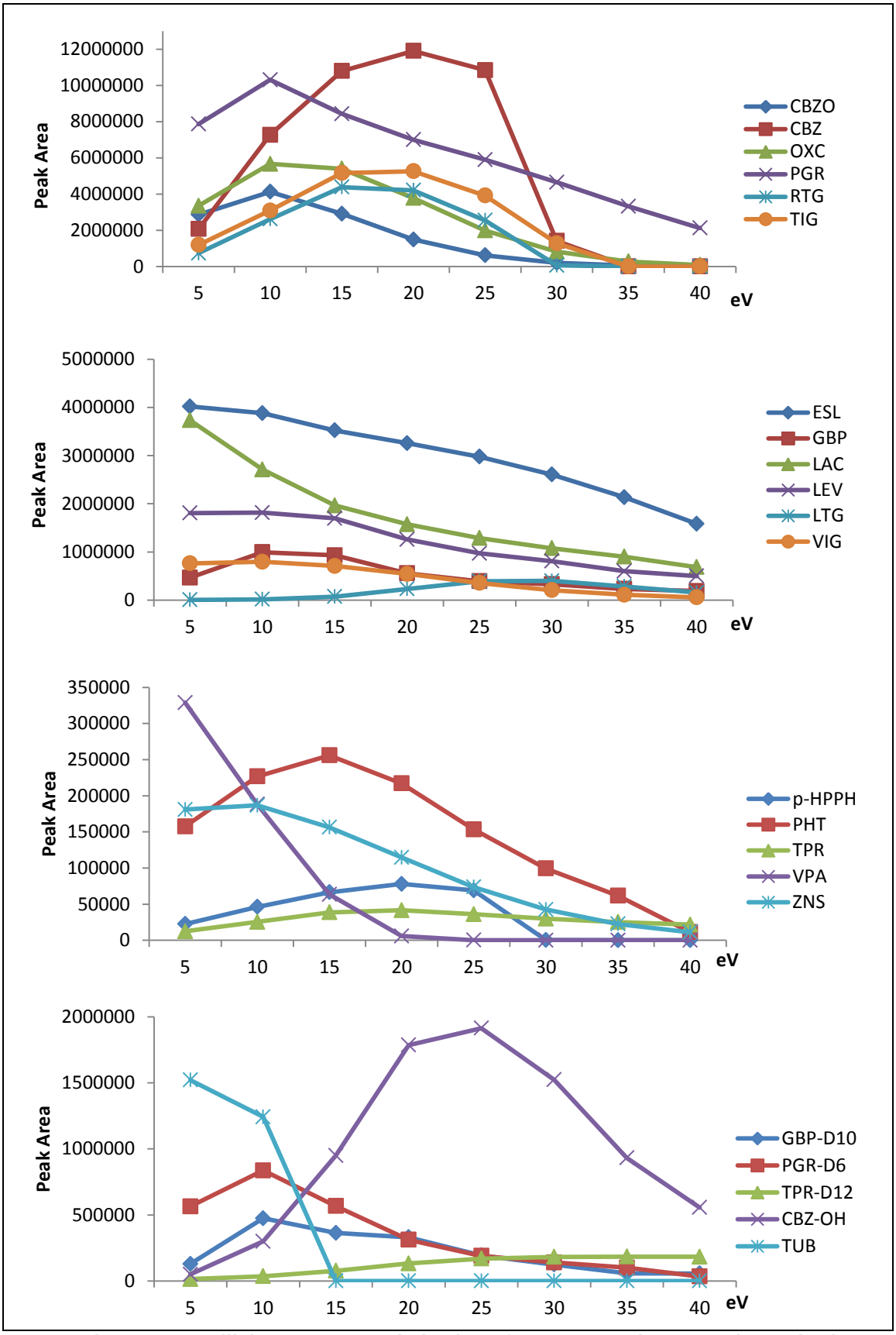


Figure 2-3: Collision Energy Optimization of 17 AEDs and 5 Internal Standards.

Table 2-13: Optimization Parameters Using 2 Different Tuning Methods.

AEDs	Ionization Mode	Precursor (m/z)	Optimizer Software				Sample Injection System			
			Quantifier (m/z)	Qualifier (m/z)	Frag[a] (V)	CE[b] (eV)	Quantifier (m/z)	Qualifier (m/z)	Frag[a] (V)	CE[b] (eV)
CBZ	+	237.3	99.1	n/a	100	20	194	179.1	140	20
CBZO	+	253.1	59.2	n/a	45	8	236.1	180	110	10
ESL	+	297.2	237.2	194.2	85	4	237.2	194.2	80	5
GBP	+	172.2	154.2	137.1	70	12	154.2	137.1	80	10
LAC	+	251.1	59.2	n/a	75	12	108.1	91.1	80	10
LEV	+	171.1	126.1	154.1	90	12	126.1	154.1	80	10
LTG	+	256.1	153.7	n/a	80	20	165.7	211	110	20
OXC	+	253.2	59.2	n/a	100	12	208	180	110	10
PGR	+	160.2	142.2	55.2	80	8	142.2	97.2	80	10
PHT	-	251.2	n/a	n/a	n/a	n/a	180	208.2	110	15
p-HPPH	-	267.2	n/a	n/a	n/a	n/a	118.1	224.1	80	20
RTG	+	304.2	n/a	n/a	n/a	n/a	230.1	258.1	110	20
TIG	+	376.1	263	n/a	110	12	149	278.2	140	20
TPR	-	338.1	n/a	n/a	n/a	n/a	78	96	140	20
VIG	+	130.1	71	113.1	70	12	113.1	71	50	10
VPA	-	143.1	143.1	n/a	100	0	143.1	n/a	80	0
ZNS	-	211.2	n/a	n/a	n/a	n/a	118.1	147.1	80	10
Internal Standards										
CBZ-DiOH	+	239.1	n/a	n/a	n/a	n/a	179	n/a	140	20
GBP-D_{10}	+	182.1	164.3	n/a	70	12	164.2	n/a	110	15
PGR-D_6	+	166.1	148.2	n/a	145	8	148.2	n/a	110	20
TPR-D_{12}	-	350.1	n/a	n/a	n/a	n/a	96	n/a	140	20
TUB	-	269.2	170	n/a	120	10	170	n/a	110	5

a: Fragmentor voltage, b: Collision energy.

2.3.2 Optimization of Nebulizer Gas Pressure

Figure 2-4 shows an increase in AEDs response when increasing the nebulizer pressure from 15 to 25 except for RTG which showed a decrease in its sensitivity. Increasing the pressure to 35 PSI decreased the abundance of all AEDs and internal standards.

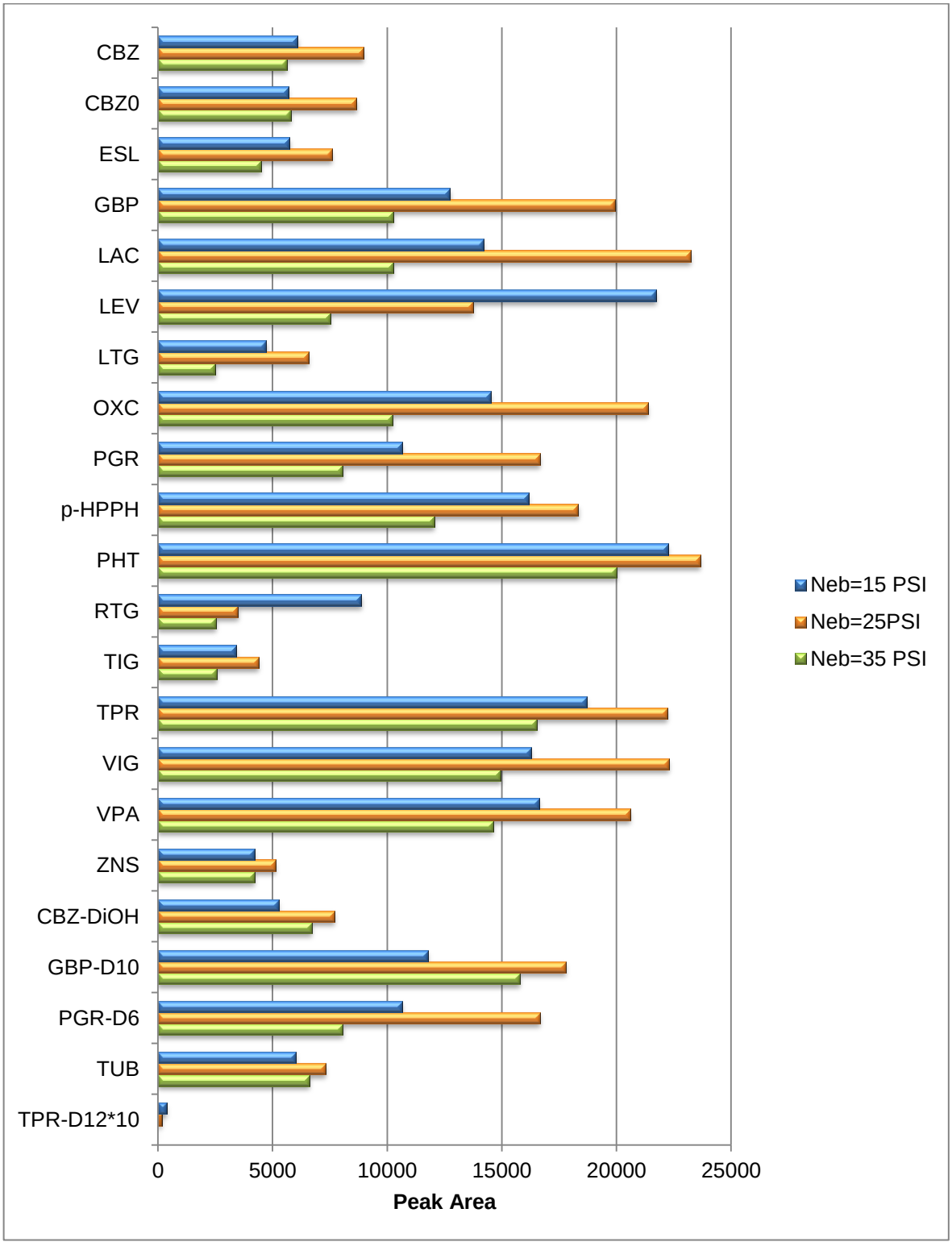


Figure 2-4: Nebulizer Gas Pressure Optimization of 15 AEDs.

TPR-D_{12} showed very low sensitivity under all conditions. TPR-D_{12} gave similar product ions and retention time to TPR but it did not mimic TPR abundance at the same concentration (Figure 2-5).

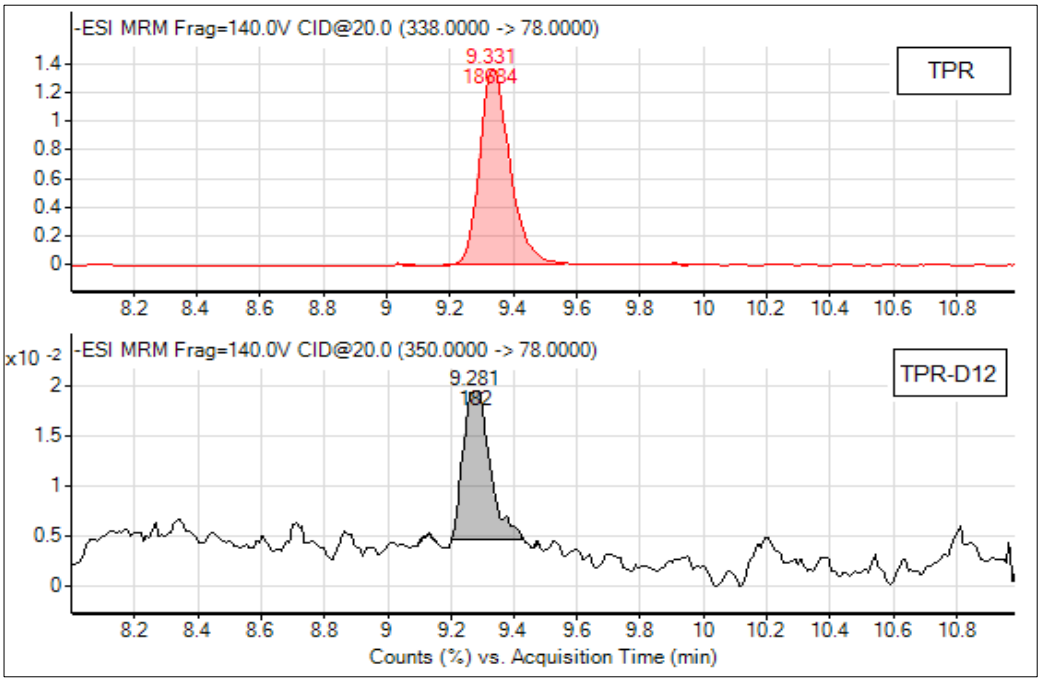


Figure 2-5: TPR and TPR-D_{12} Chromatograms at Concentration 10 mg/L and Neb 15 PSI.

A pressure of 25 PSI was used during the method development period. However, at a later stage, the gas pressure was decreased to 15 PSI in order to obtain good sensitivity for all drugs including RTG. Furthermore, decreasing the nebulizer pressure decreased the sensitivity slightly which helped to prevent the MS source saturating due to high calibrator concentrations. On the other hand, TPR-D_{12} was excluded from this project due to its poor abundance under the used conditions. Also, PGR-D_6 and GBP-D_{10} have similar structure and same retention time. As a result, it was decided to use GBP-D_{10} instead of PGR-D_6 due to its cheaper cost.

2.3.3 Optimization of Mobile Phase

2.3.3.1 Aqueous Phase Additives

Four buffers with different pH ranging from 2.8 to 9.3 were optimized using the same gradient system which is illustrated in Figure 2-1. ACN was used as an organic phase in all of these. Taking into account the sensitivity, relative abundance, precursor ion fragmentation and enhancing product ion formation, 10 mM Ammonium acetate (pH= 5)

was the mobile phase which gave the optimum result for most of the AEDs under the same analysis conditions (Figure 2-6, Figure 2-7 and Figure 2-8).

It was obvious that highly acidic mobile phase such as 0.1% formic acid (pH=2.8) had a strong suppression effect on product ion formation of PHT, TPR, VPA and ZNS which are analysed in negative ionization mode (Figure 2-7) whereas it enhanced the sensitivity of other drugs analysed in positive mode such as gabapentin, pregabalin and lacosamide. Similar results were observed with 10mM ammonium formate (pH= 3). Although ammonium formate and ammonium acetate have the same concentration of 10 mM, ammonium acetate exhibited higher responses with all AEDs and their internal standards. That may be due to the difference in pH of the mobile phase from 7 (ammonium formate) to 5 (ammonium acetate) which improves the ionization and increases the analyte sensitivity. Regarding 10mM ammonium carbonate, its high pH exhibited suppressive effects with most AEDs except OXC, LEV and LTG which showed considerable responses but still lower than 10mM ammonium acetate. However, amphoteric drugs and their deuterated internal standards; GBP, PGR and VIG showed good sensitivity with either acidic or basic mobile phases. PGR and its internal standard PGR-D_6 gave slightly higher responses with 0.1% formic acid compared to 10 mM ammonium acetate. Results were presented using the average peak area.

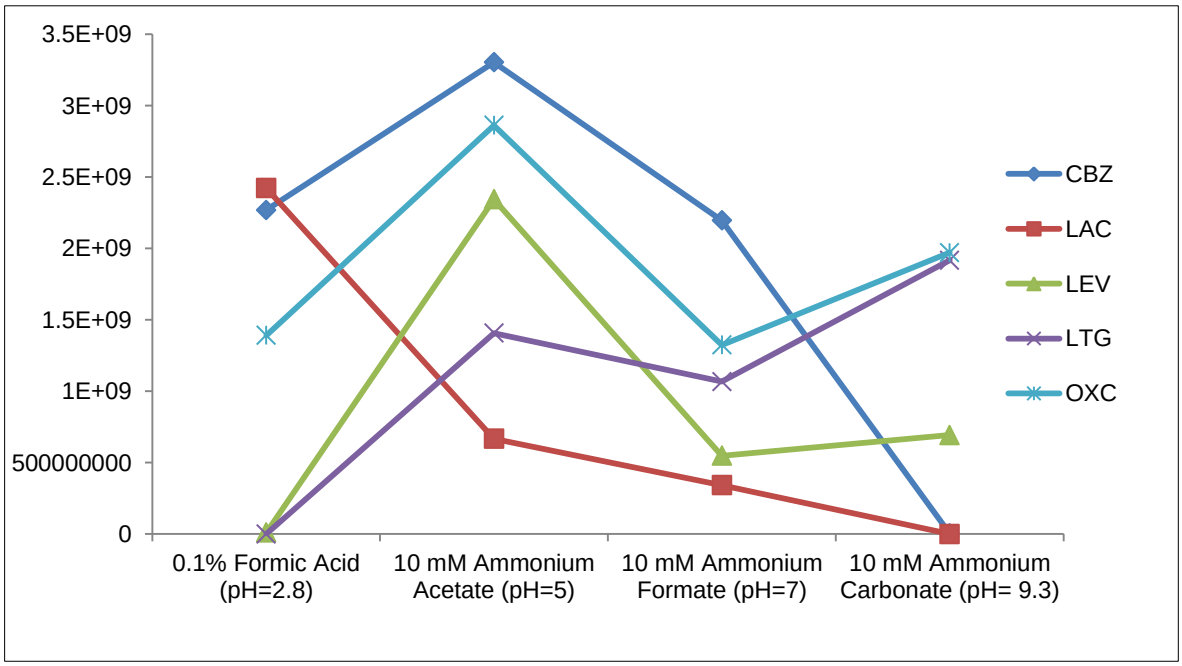


Figure 2-6: Effect of Mobile Phase Additives on Analyte Abundance in Positive Ionization Mode.

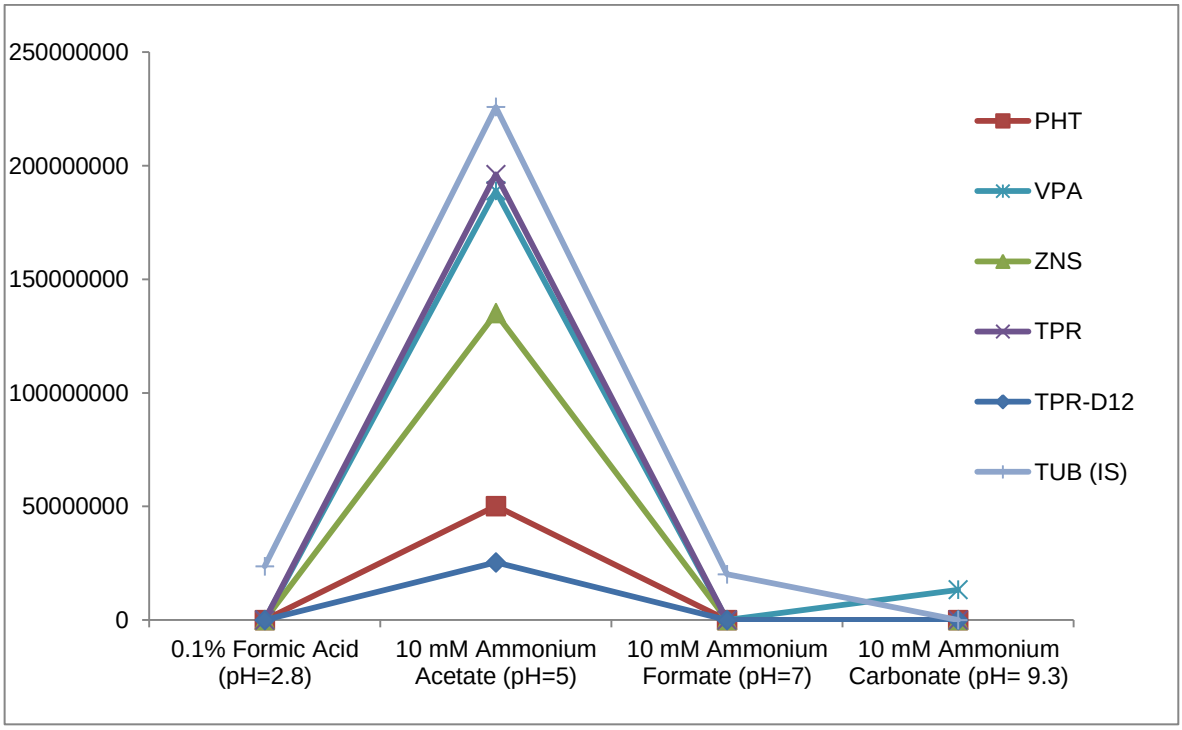


Figure 2-7: Effect of Mobile Phase Additives on Analyte abundance in Negative Ionization Mode.

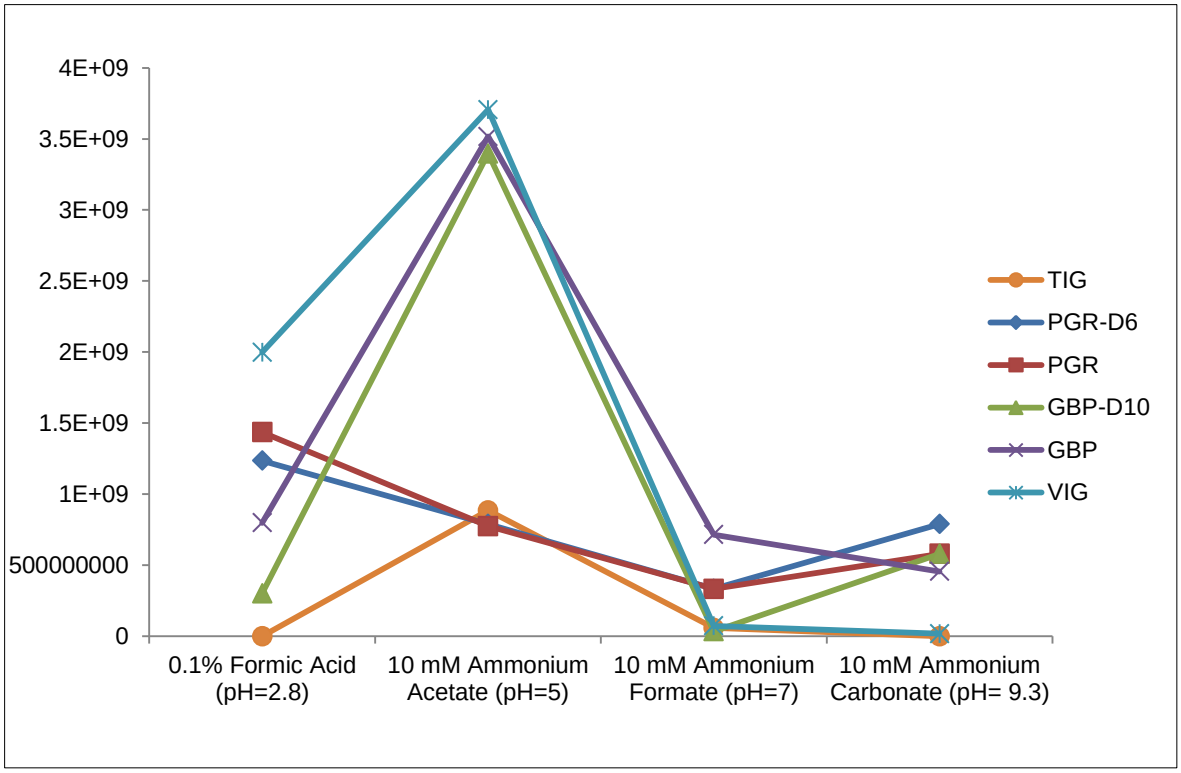


Figure 2-8: Effect of Mobile Phase Additives on Amphoteric Analyte Abundance in Positive Ionization Mode.

As a result, 10 mM ammonium acetate is considered the mobile phase of choice that showed acceptable sensitivity for 13 AEDs and 4 internal standards.

2.3.3.2 Mobile Phase pH

Adjustment of pH of 10 mM ammonium acetate from 6.8 to 5.5, 4.5 and 4 showed no improvement effect on resolution or ionization. On the other hand, using mobile phase with low salt and acid concentration gave higher responses and improved the sensitivity of the analytes except TPR-D_{12} which did not show a good response with any of the conditions tested above (Figure 2-9). As a result, a mobile phase with a pH of 6.8 was used.

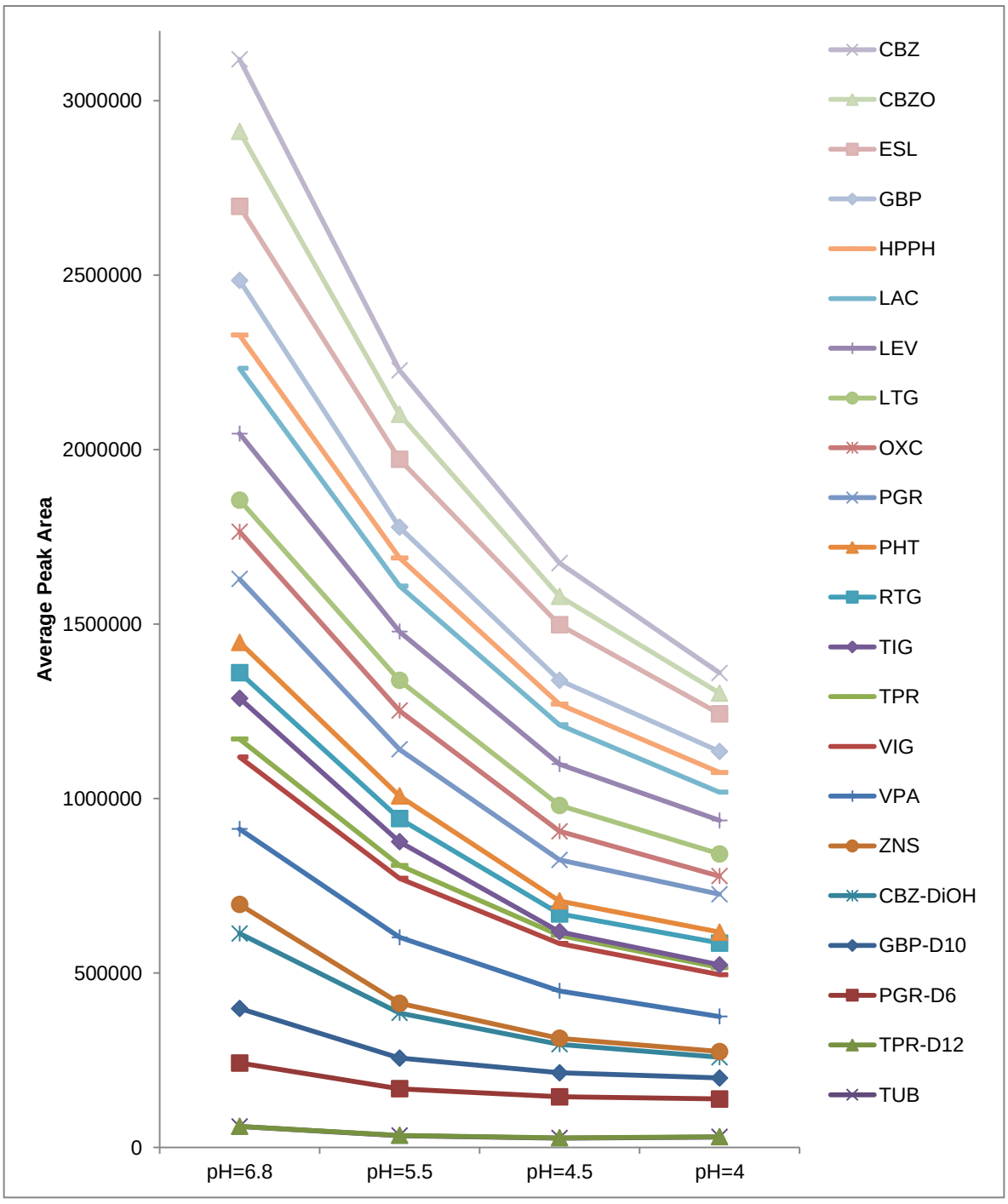


Figure 2-9: Effect of Acetic Acid as an Additive in Aqueous Mobile Phase on AEDs Response.

2.3.3.3 Aqueous Phase Molarity

It was clear that decreasing the concentration of the additive salt from 10 mM to 2 mM resulted in a higher response and better sensitivity for all the analytes especially for those tested in negative mode such as PHT and TPR. Thus, it was decided to use 2mM ammonium acetate as an aqueous mobile phase (Figure 2-10). Data is not available for PGR-D_6 and TPR-D_{12} because they were excluded from the project at this stage (see 2.3.2).

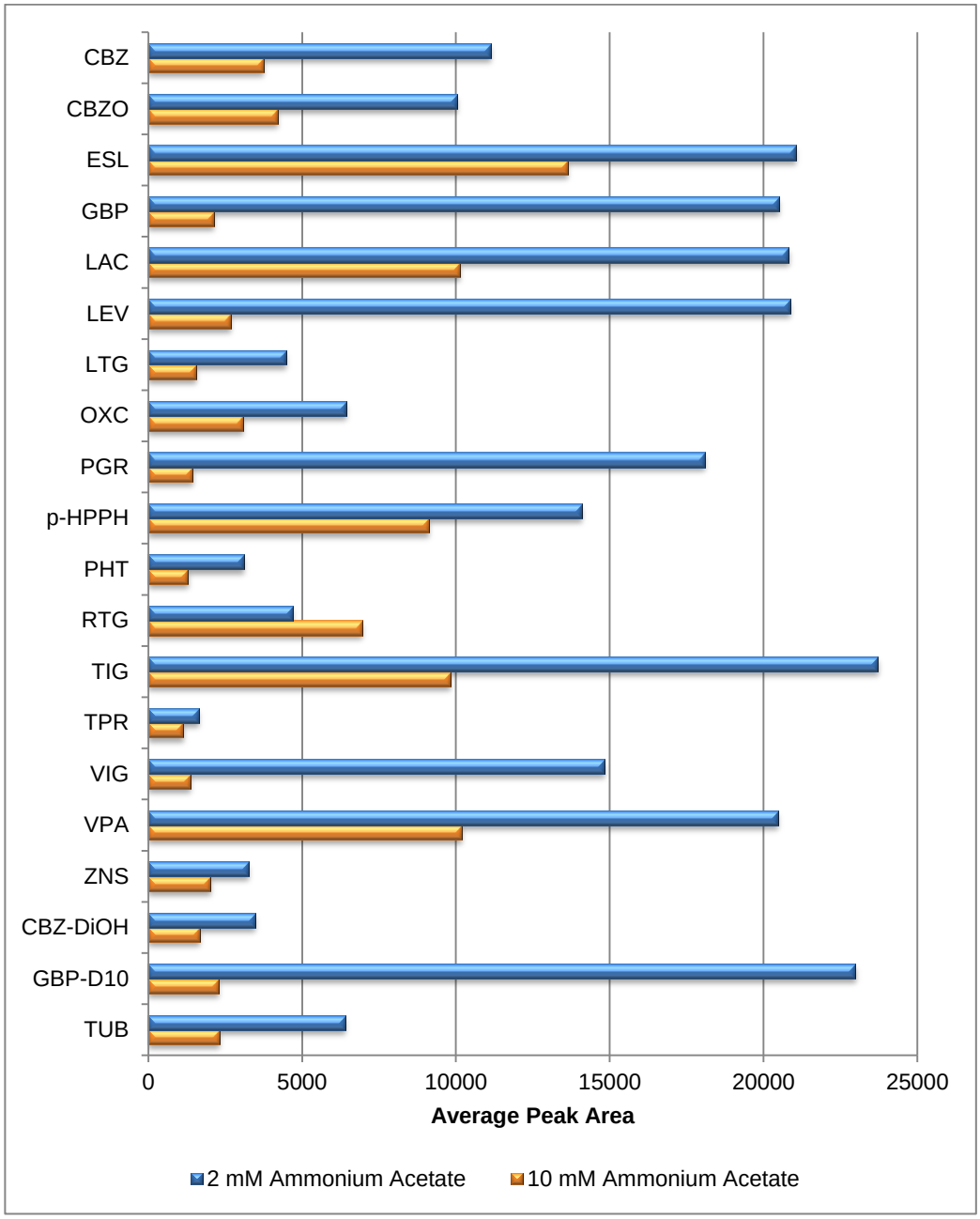


Figure 2-10: Effect of Ammonium Acetate Concentration on AED Responses.

2.3.3.4 Organic Phase

Methanol was shown to give higher responses with all AEDs compared with acetonitrile with the exception of RTG which exhibited a slightly higher response using acetonitrile (Figure 2-11). Although acetonitrile gave a better peak shape it also exhibited a suppressive effect on product ion formation which in turn affected the lower limit of detection (LOQ) for some drugs such as PHT, p-HPPH, LTG, OXC and ZNS. Methanol and acetonitrile effects on the LOQ for all AEDs and internal standards are illustrated in Figure 2-12, Figure 2-13 and Figure 2-14.

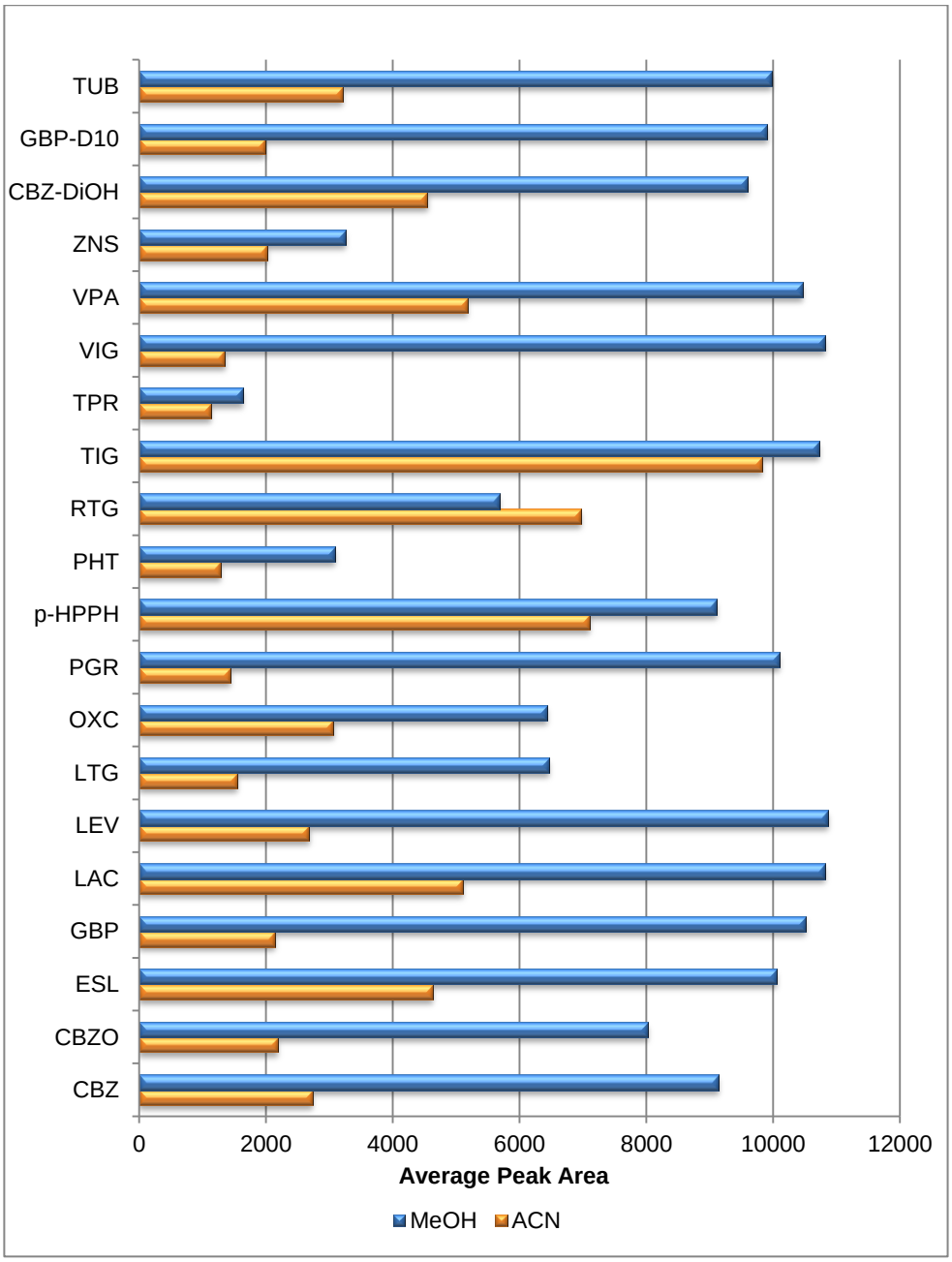


Figure 2-11: Comparison of Response of AEDs and Internal Standards Using MeOH and ACN in Mobile Phase.

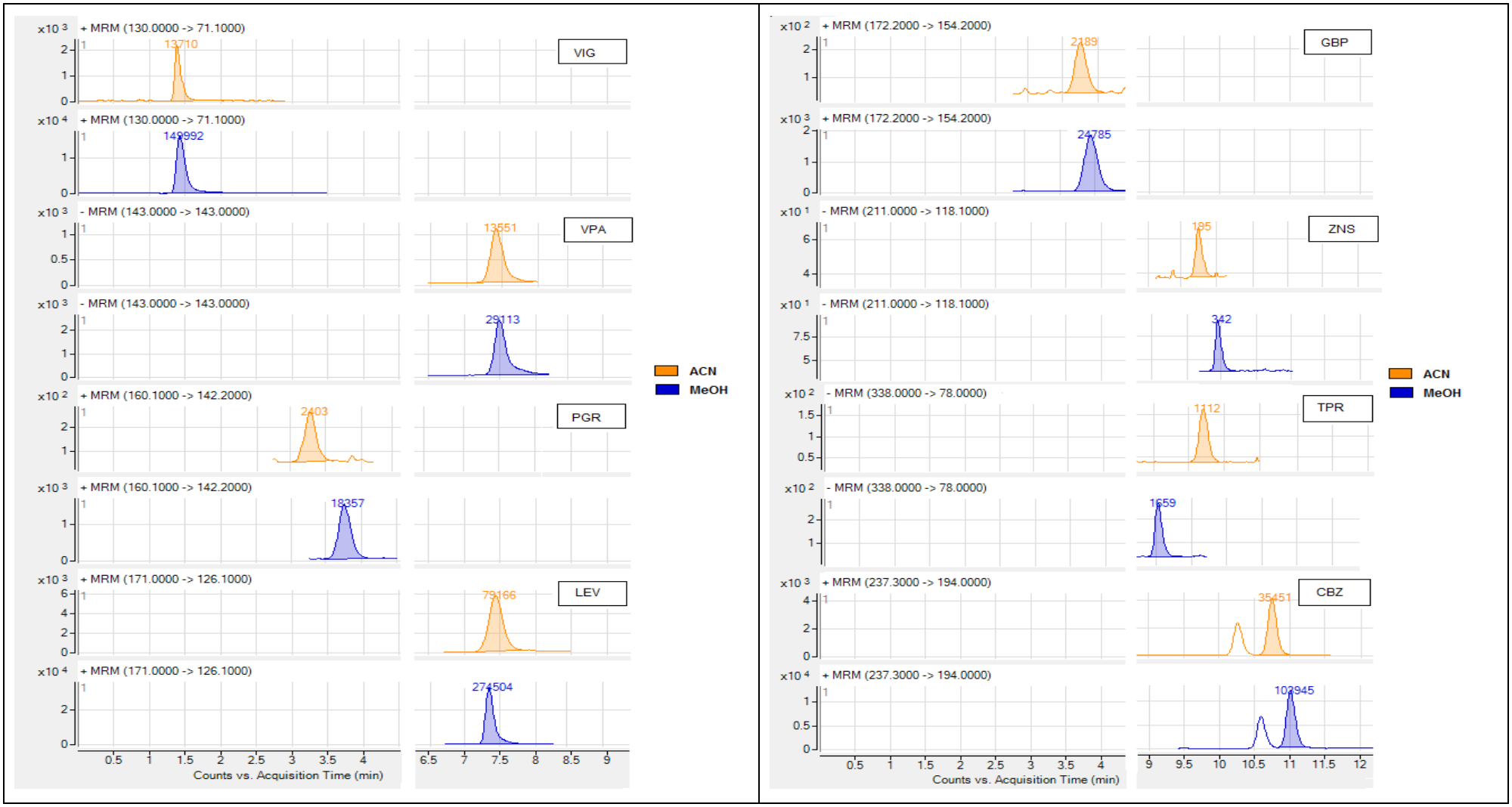


Figure 2-12: Effects of MeOH and ACN as an Organic Mobile Phase on LOQ.

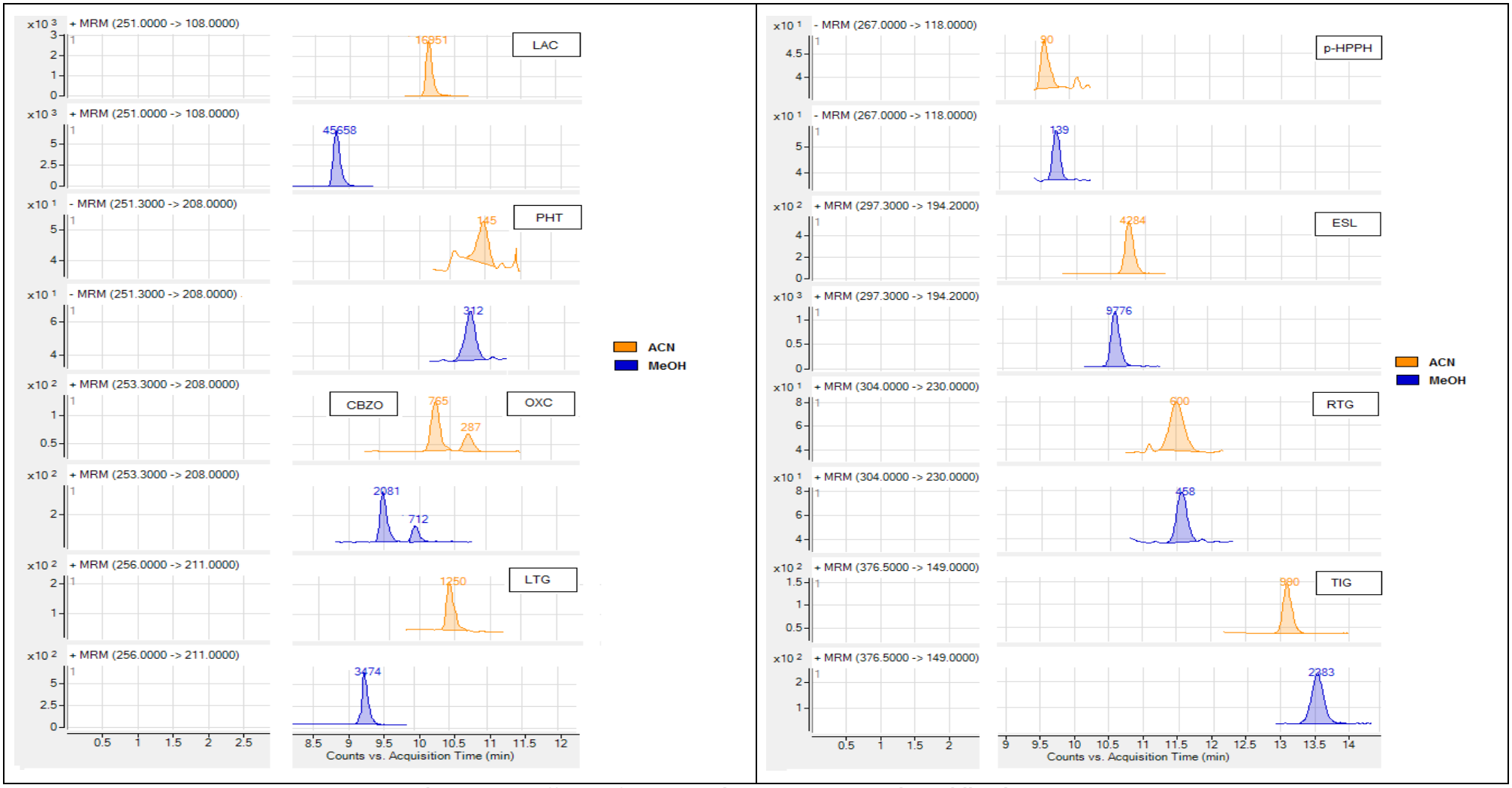


Figure 2-13: Effects of MeOH and ACN as an Organic Mobile Phase on LOQ.

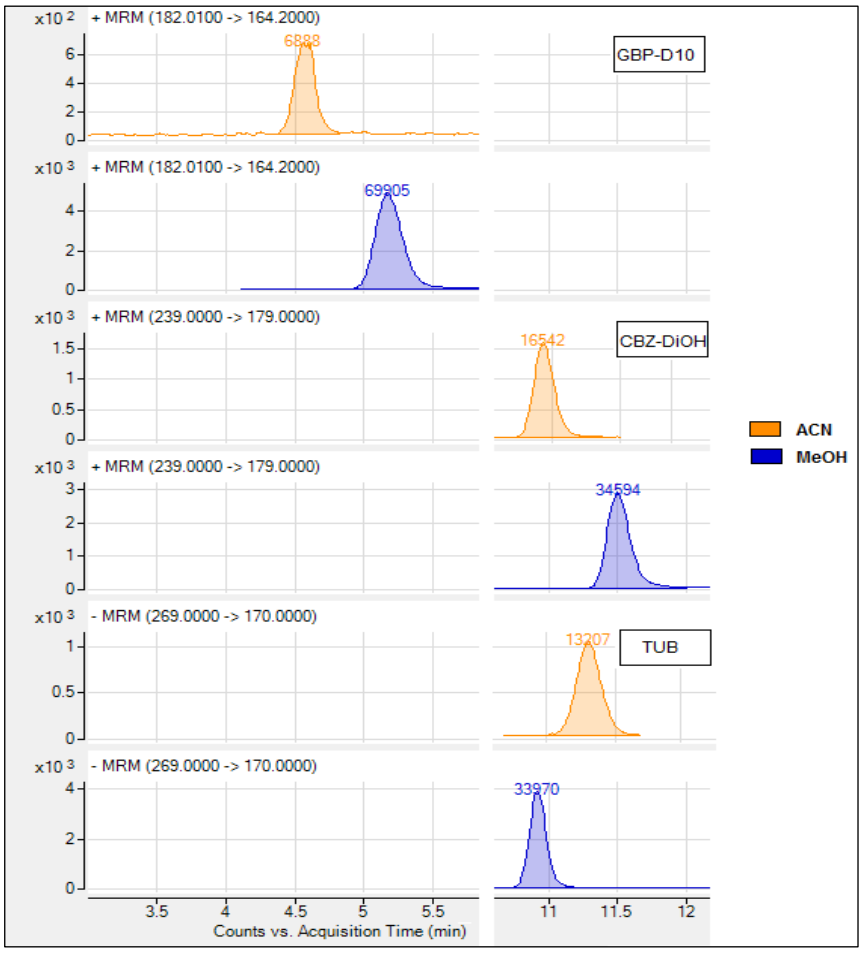


Figure 2-14: Effects of MeOH and ACN as an Organic Mobile Phase on Internal Standards.

Finally, 2 mM ammonium acetate in water (pH not adjusted) and 2 mM ammonium acetate in methanol were used as the mobile phase of choice for all AEDs of interest and their internal standards.

2.3.4 Optimization of LC Column Stationary Phase and Temperature

2.3.4.1 Stationary Phase

Figure 2-15 to Figure 2-17 show the response of the analytes using 4 different columns. It was clear that using the Gemini C18 column (150 mm x 2.0 mm, 5 µm) gave a greater response and better peak shape for every analyte except PHT and its metabolite p-HPPH which showed higher abundance using the Agilent C8 XDB column (150 mmx 4.6 mm x 5µm).

Although the Agilent C8 showed a higher abundance with some drugs, the peak shape was unacceptable. They exhibited wide and tailing peaks for most drugs. On the other hand, due to its wide diameter, the flow rate had to be increased from 0.3 µL/min to 0.6 µL/min in order to shorten the analysis time from 40 to 17 minutes. This flow rate caused a quick

build up on the front of the source which decreased the sensitivity of the analysis and increased the frequency of the MS source maintenance. Moreover, it did not give good separation results with CBZ, OXC, ESL and CBZO. The same issue was seen when using Obelisc R (150 mm x 4.6 mm, 5 µm) due to its wide diameter as well. However, Obelisc R showed good separation with CBZ and ESL but not OXC and CBZO. On the other hand, Obelisc R did not give the expected results with most drugs such as VPA, LTG and, CBZ-DiOH.

The Agilent C18 XDB (50 mm x 4.6 mm, 1.8 µm) gave very sharp peaks and good responses with all the drugs due to its small particle size. However, CBZ derivatives separation was not successful. Furthermore, the very small particle size imposed a high pressure on the LC pump which can shorten the pump life time on a longer term basis. Additionally, columns with small particle size are not preferable for postmortem blood analysis due to the complicated and clotted sample nature which can cause a quick build up in the column if the extraction procedure is not sufficiently clean. As a result, Gemini C18 column (150 mm x 2.0 mm, 5 µm) was chosen as column of choice to develop this method.

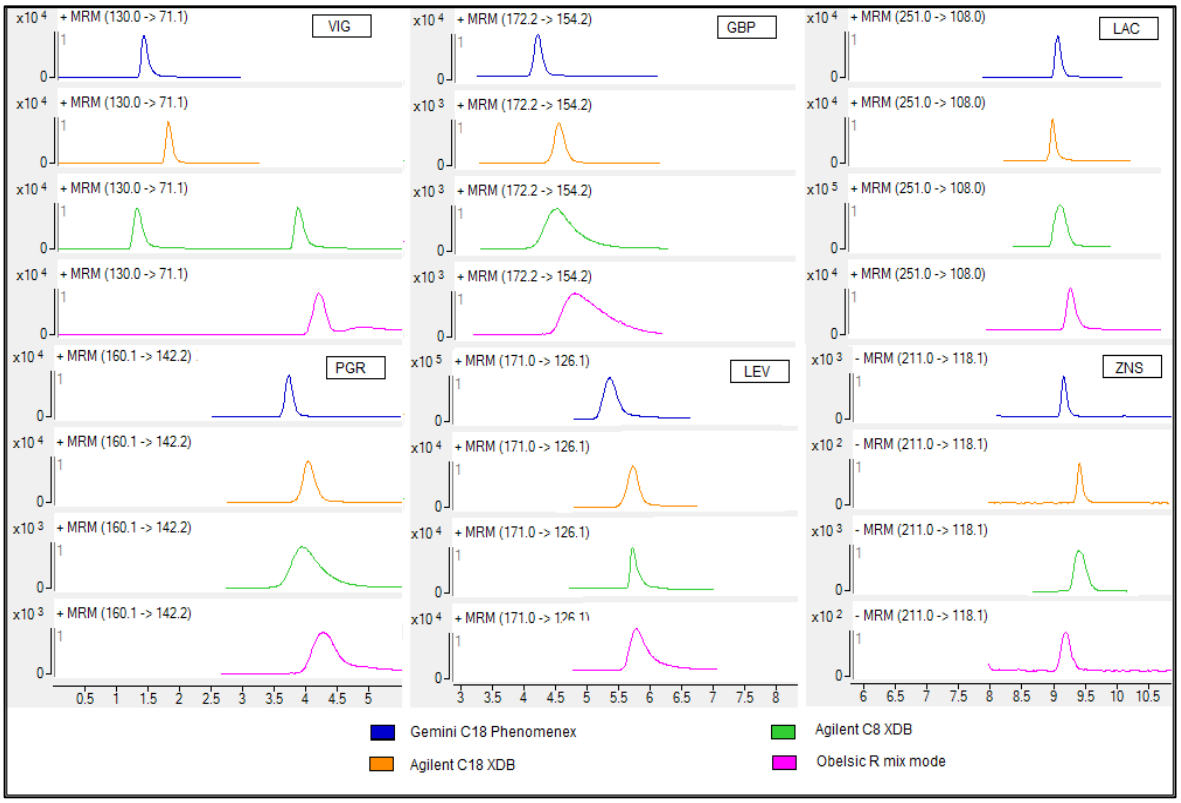


Figure 2-15: Column Diameter and Stationary Phase Effect on Analyte Peak Shape and Resolution.

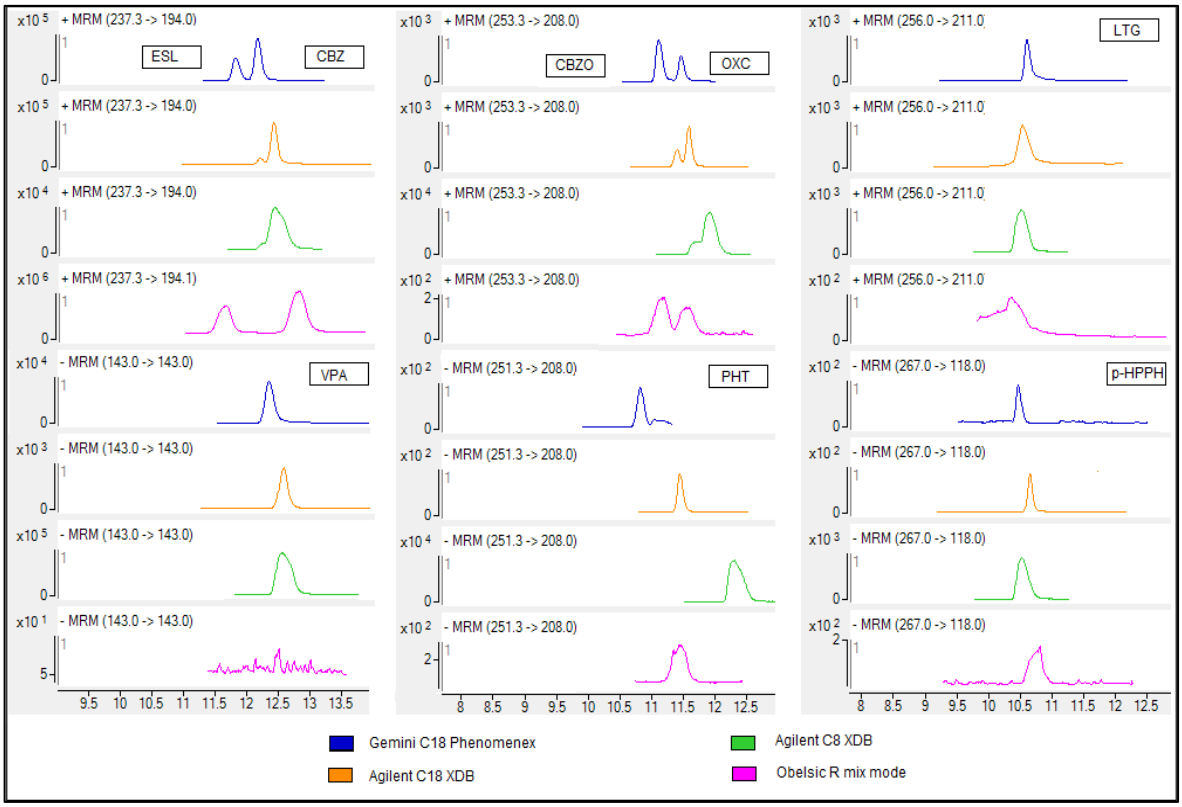


Figure 2-16: Column Diameter and Stationary Phase Effect on Analyte Peak Shape and Resolution.

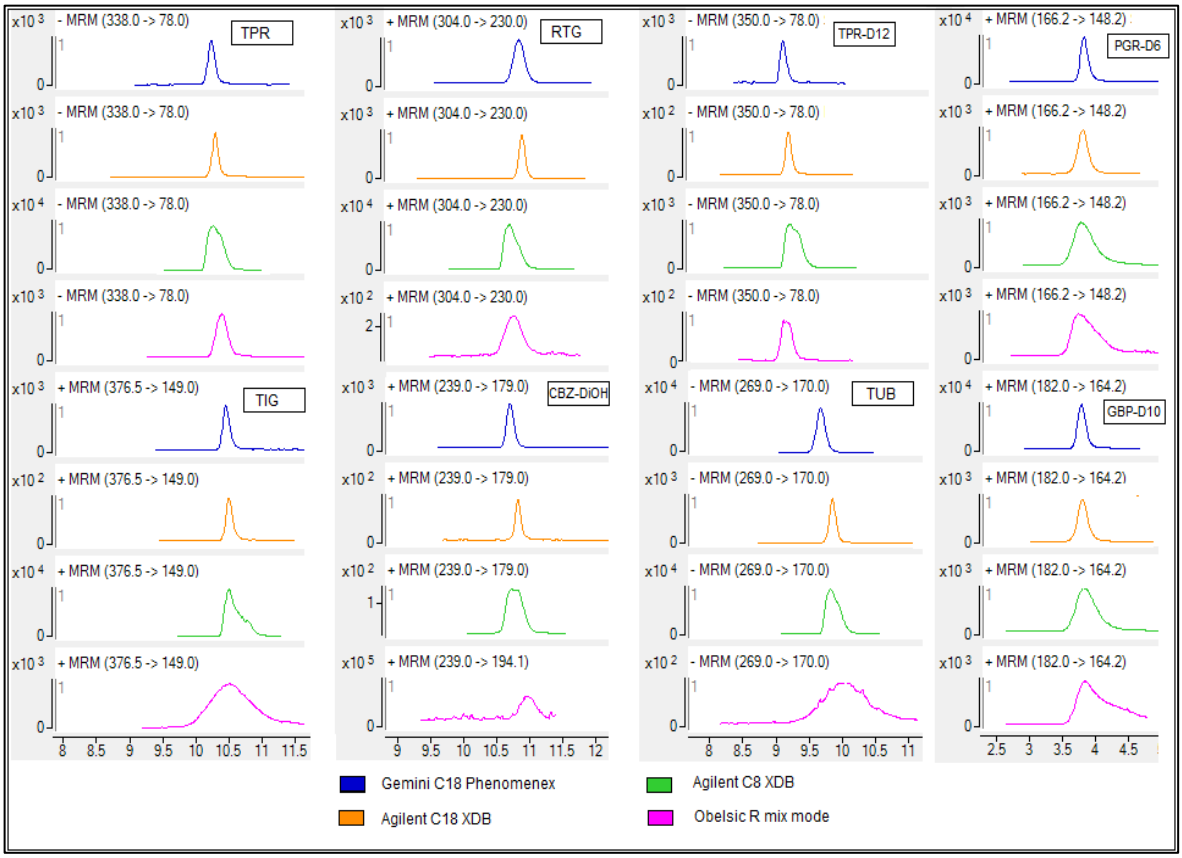


Figure 2-17: Column Diameter and Stationary Phase Effect on Analyte Peak Shape and Resolution.

2.3.4.2 LC Column Temperature

After choosing the Gemini C18 column (150 x 2.1 mm, 5 µm) from Phenomenex and 2 mM ammonium acetate in water/2 mM ammonium acetate in methanol as the most suitable mobile phase the column temperature was optimized. It was found that increasing the column temperature from 25 to 40°C showed a slight increase in the method sensitivity by increasing the AEDs intensities (Figure 2-18)

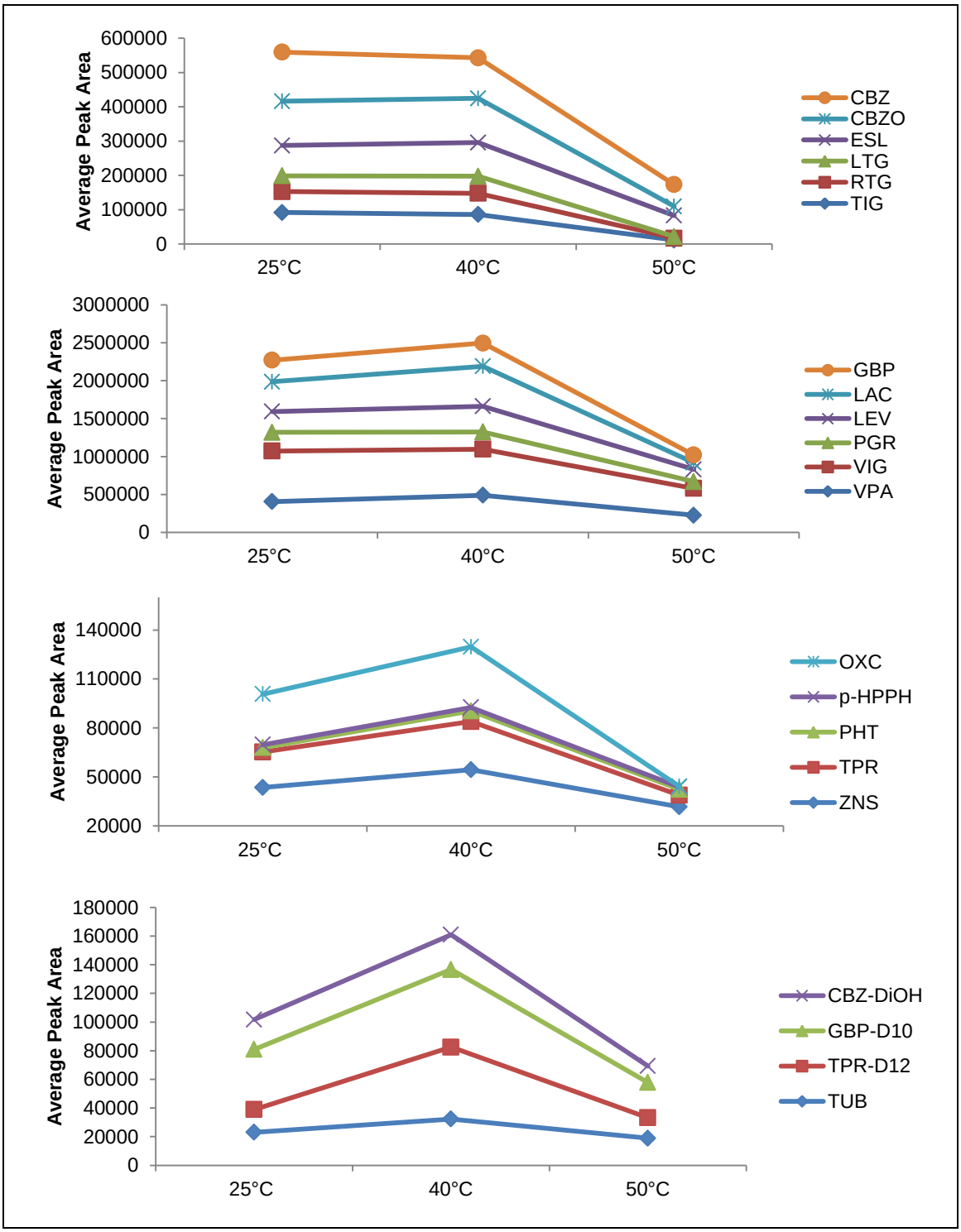


Figure 2-18: Effect of Chromatographic Column Temperature on AEDs and Internal Standards Sensitivity.

Furthermore, it improved the instrument performance by decreasing the turbo pump pressure as a result of decreasing the mobile phase viscosity. However, at 50°C, the sensitivity of all the drugs and internal standards were significant reduced. The column temperature was therefore set at 40°C.

2.3.5 Optimization of Chromatographic Separation

OXC and ESL are new AEDs derived from CBZ whereas CBZO is the metabolite of CBZ. All these compounds are structurally similar (Figure 2-19). OXC and CBZO have identical fragmentation patterns. Their product ions are 236, 210, 208 and 180 m/z.

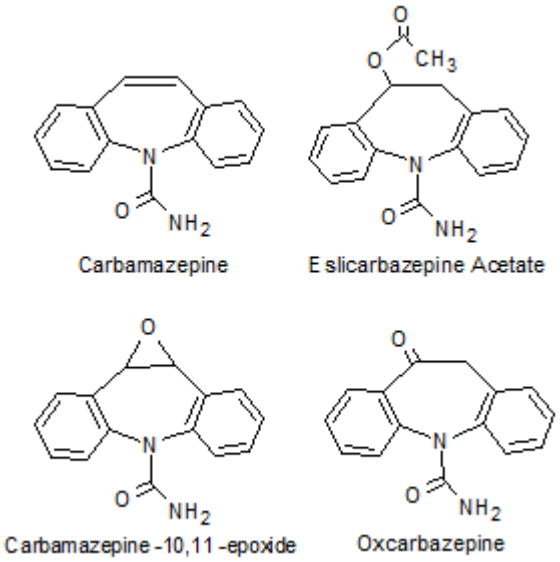


Figure 2-19: CBZ, CBZO, OXC and ESL Chemical Structure.

Although the Gemini C18 column gave good separation of these 4 drugs, the separation was very sensitive to any change in the mobile phase composition or flow which led the peaks to be merged again.

Slowing the flow rate from 0.3 mL/min to 0.2 mL/min to 0.1 mL/min gave a partial separation for OXC and CBZO but there was no improvement to CBZ and ESL separation (Figure 2-20). On the other hand, reducing the flow rate increased the method run time from 17 to 32 minutes in order that all the drugs could be eluted.

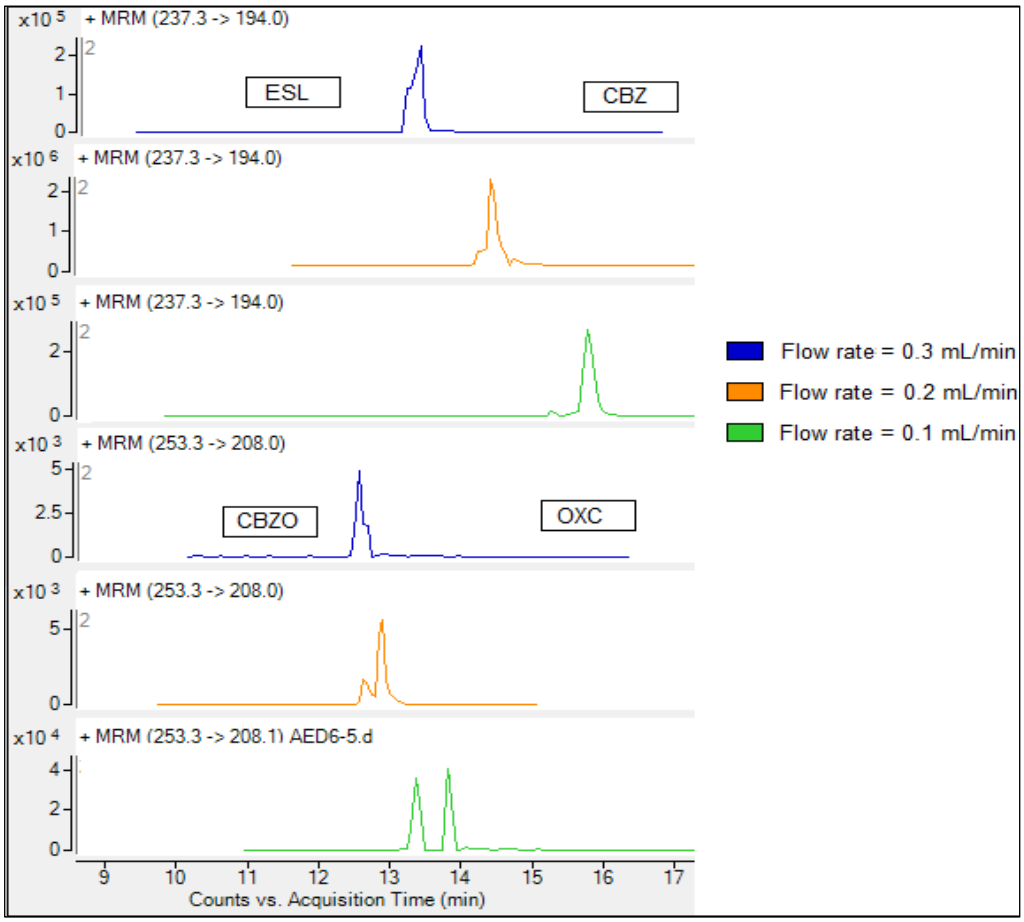


Figure 2-20: Flow Rate Effect on CBZ Derivatives Separation.

The change of the gradient showed a significant change in the separation and retention time of these 4 drugs as illustrated in Figure 2-21. Five different systems were used as detailed in Table 2-9. Best results were obtained with system 2 and 5. Tthe gradient mobile phase system implied finally started at 80:20 A/B and was increased to 50:50 A/B within 2 minutes. This percentage was maintained for 6 minutes before it was increased to 10:90 A/B for 2 minutes. The percentage decreased finally to 80:20 A/B for 7 minutes in order to condition the column before the next injection.

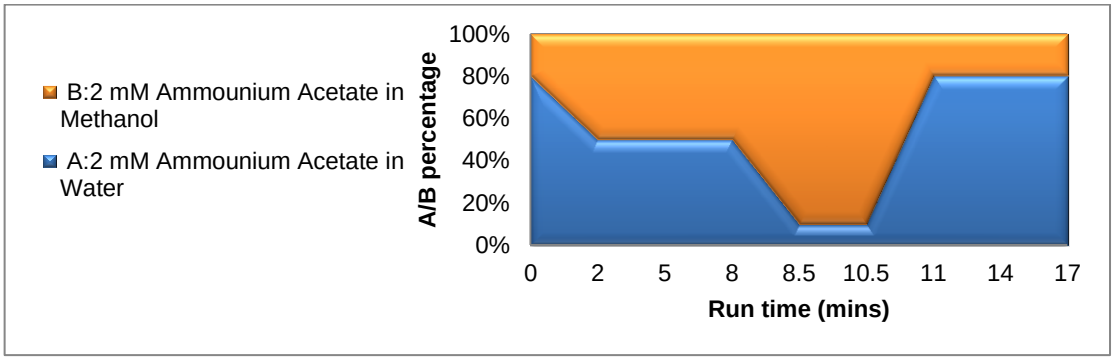


Figure 2-21: Gradient System Used to Improve the Separation of 4 AEDs.

The change in the aqueous/organic phase percentage affects the polarity of the mobile phase and greatly improves the drugs separation as showed in Figure 2-22.

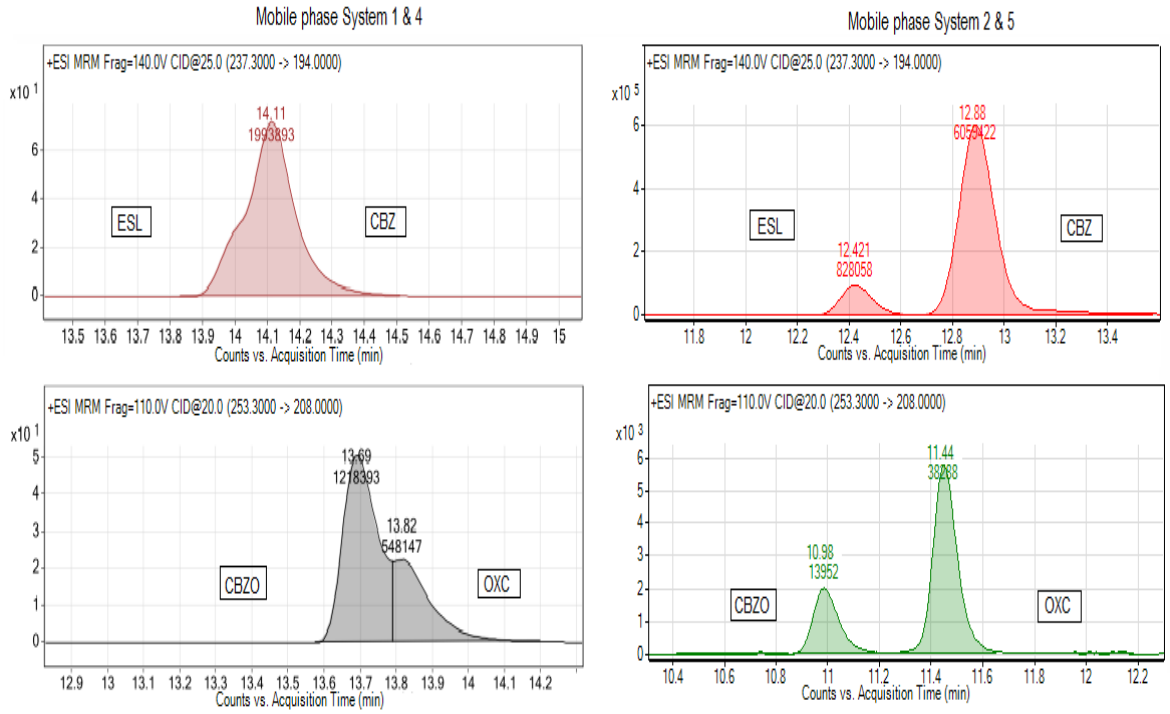


Figure 2-22: Carbamazepine (CBZ) and Eslicarbazepine (ESL), Oxcarbazepine (OXC) and Carbamazepine Epoxide (CBZO) Separation Using Different Mobile Phase Gradient Systems.

Finally, Figure 2-23 shows the chromatogram of all analytes of interest obtained by dynamic multiple reaction monitoring mode and using non-extracted standards mixture at a concentration of 10 mg/L.

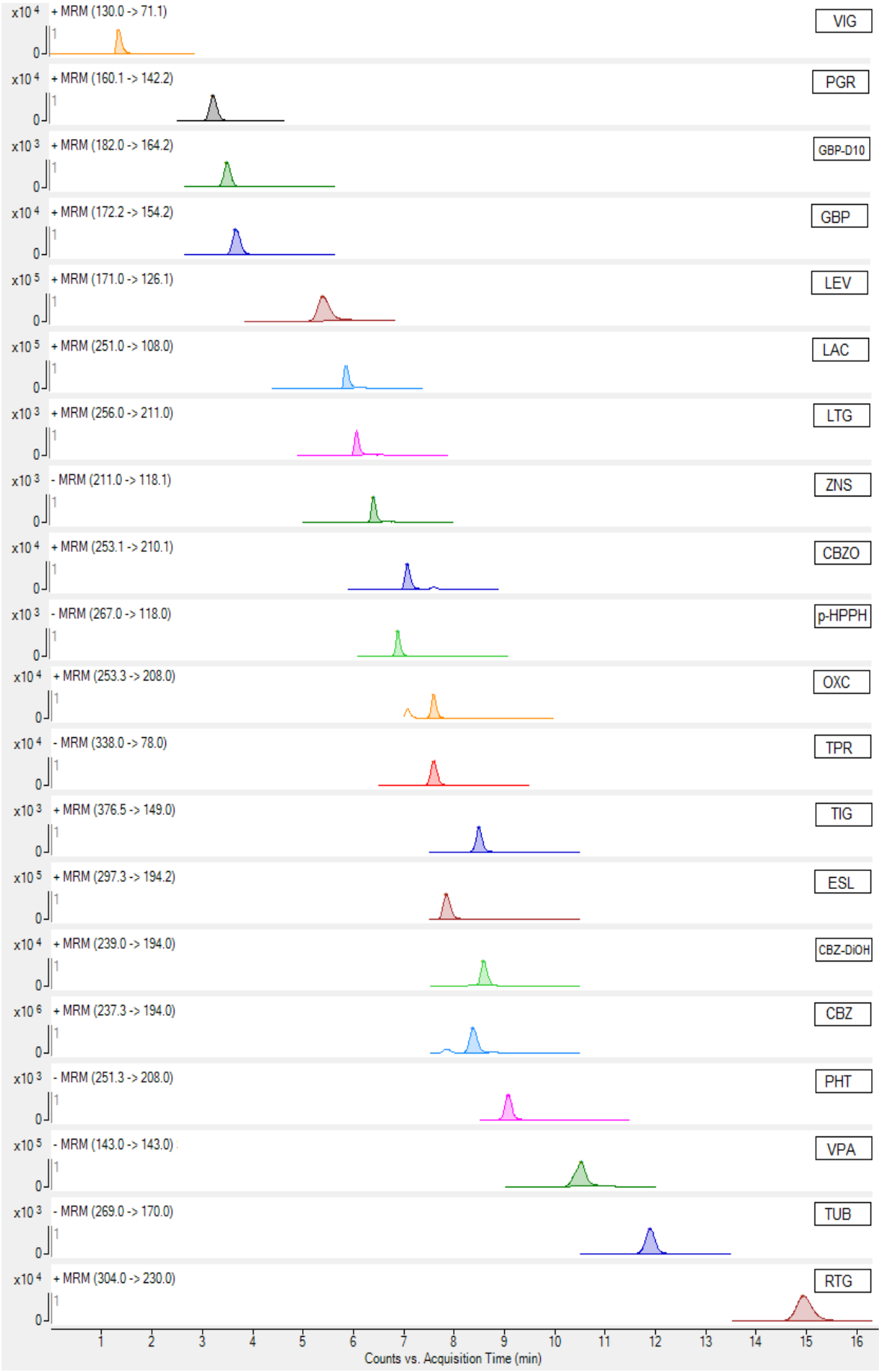


Figure 2-23: The Chromatogram of 17 AEDs and 3 Internal Standards Using a Standard Mixture at 10mg/L.

2.3.6 Extraction Optimization - Solid Phase Extraction vs Protein Precipitation

Solid phase extraction did not achieve the optimum results for all analytes although the used cartridges are designed for the extraction of basic and acidic drugs mixtures. Peak shape and sample purity were not as good as protein precipitation and blood samples following SPE extraction were brownish in colour (Figure 2-24).

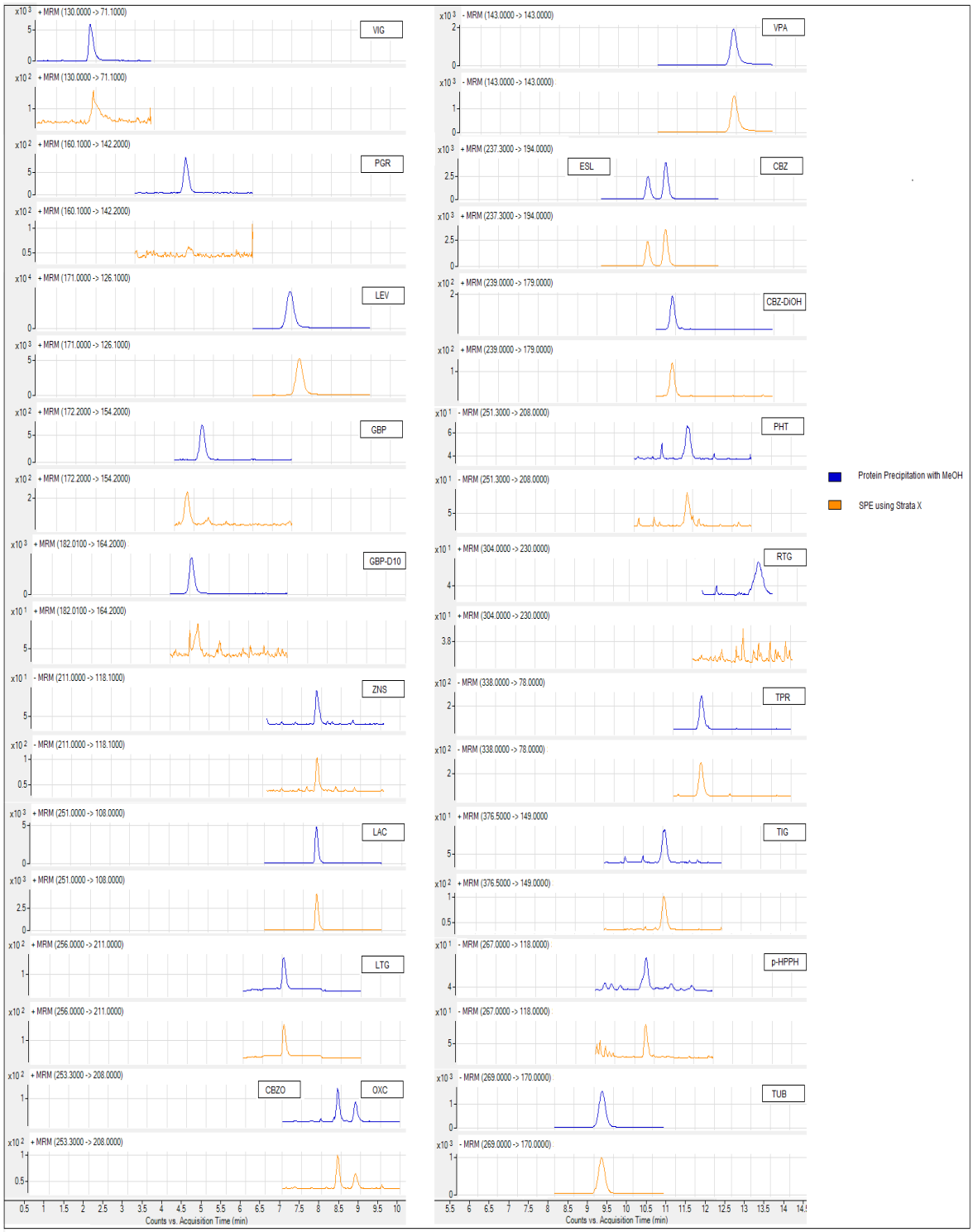


Figure 2-24: Chromatogram of Extracted Analytes Using SPE and Protein Precipitation at a Concentration of 10 mg/L.

Amphoteric drugs (GBP, PGR and VIG) showed poor recovery using the SPE method (43.4%, 4.3% and 4.6% respectively) and RTG could not be extracted at all. Protein precipitation attained recoveries higher than 70% for all the analytes and their internal standards except RTG which was less than 50% (Table 2-14). Regarding linearity, the R^2 values were not acceptable for some drugs using SPE due to their poor recoveries (amphoteric drugs). For other drugs such ESL, LEV, LTG and ZNS, the upper limit of quantification (ULOQ) was not high enough compared to protein precipitation. This would therefore require reanalysis and dilution of real case samples in order to achieve accurate quantitative results within the linear range. Furthermore, SPE was found to be time consuming due to the number of steps involved with sample preparation.

Table 2-14: Recoveries, Calibration Ranges and Linearity Values for SPE and Protein Precipitation.

AEDs	Recovery (%)		Calibration Range (mg/L)		Calibration Model		R^2	
	P.P	SPE	P.P	SPE	P.P	SPE	P.P	SPE
CBZ	103.3	110.5	0.5-50	0.5-50	Quadratic	Quadratic	0.999	0.996
CBZO	116.0	85.0	0.5-50	0.5-50	Quadratic	Quadratic	0.999	0.997
ESL	94.2	118.0	0.5-50	0.5-25.0	Quadratic	Linear	0.999	0.991
GBP	89.3	43.4	0.5-50	N/A	Quadratic	N/A	0.998	N/A
LAC	100.9	77.7	0.5-50	0.5-50	Quadratic	Quadratic	0.999	0.998
LEV	100.4	38.2	5.0-300	5.0-200.0	Quadratic	Quadratic	0.999	0.999
LTG	110.3	61.3	1.0-50	1.0-25.0	Quadratic	Linear	0.9995	0.995
OXC	104.3	77.0	0.05-10	0.05-10	Linear	Linear	0.999	0.997
PGR	104.6	4.3	0.5-50	N/A	Quadratic	N/A	0.997	N/A
PHT	100.0	85.9	0.5-50	1.0-50.0	Linear	Linear	0.9997	0.998
p-HPPH	75.2	82.4	0.5-50	0.5-50	Linear	Linear	0.998	0.994
RTG	50.0	0.3	0.5-10	N/A	Quadratic	N/A	0.991	N/A
TIG	85.4	100.2	0.05-10	0.05-10	Quadratic	Linear	0.998	0.997
TPR	90.0	105.0	0.5-50	0.5-50	Linear	Linear	0.997	0.997
VIG	70.9	4.6	5.0-300	N/A	Quadratic	N/A	0.999	N/A
VPA	97.4	67.1	5.0-300	5.0-100	Linear	Linear	0.997	0.988
ZNS	100.0	55.0	0.5-50	1.0-25.0	Linear	Linear	0.996	0.995

The protein precipitation method compared with the SPE method for blood was more robust and used less solvent. Linearity criteria were acceptable for all analytes ($R^2 > 0.996$) using high ULOQ concentrations, except for RTG. In spite of its adequate recovery, the methods were not sufficiently robust to give acceptable linearity for RTG. Sample preparation was fast using protein precipitation due to the fact that it contained fewer and

shorter steps. Thus, it was decided to use protein precipitation as the method of choice to extract all the analytes simultaneously.

2.3.7 Sample Reconstitution Optimization

Due to the wide range of analytes used in this method and the high abundance of the analytes, the quadratic model had to be used with most of these and a carryover issue was observed. Although the quadratic model was reported to have been used in literature, a series of dilutions were applied to the extraction supernatant in order to improve the linearity, and decrease the carryover issue while maintaining an acceptable LOQ for all the drugs. The linearity values (R^2) were > 0.99 for all dilutions. The only factors affected by dilution were LOQ and calibration model. Table 2-15 shows LOQ and calibration models resulted from applied dilutions. It was clear that increasing the dilution helps to improve the linearity by preventing the MS source from saturating due to high concentrations used. Although a 4 mL dilution gave linear ranges with all the analytes (except RTG), it also increased the LOQ for some drugs, such as LTG, PHT, p-HPPH, TPR and ZNS, due to the variance in their intensities. A 3 mL dilution gave acceptable LOQ and linear range for all drugs except LTG and solved the issue of carryover at high ULOQ concentrations, thus, it was used as the reconstitution volume.

The protein precipitation method used a total of 400 µL of methanol to 100 µL of blood. After centrifuging the sample, the supernatant volume was approximately 390 to 430 mL depending on the water volume in the blood sample. In order to simplify the extraction method and obtain accurate results, it was decided to use half of the supernatant volume, 200 µL and dilute this to 1.5 mL with mobile phase (half of the chosen dilution volume). This adjustment meant that the evaporation step could be avoided, saving time and giving better accuracy.

Table 2-15: LOQ and Calibration Models Resulting From Series of Dilutions.

AEDs	LOQ (mg/L)				Calibration Model			
	1 mL	2mL	3mL	4mL	1 mL	2mL	3mL	4mL
CBZ	0.50	0.50	0.50	0.50	Quadratic	Linear	Linear	Linear
CBZO	0.50	0.50	0.50	0.50	Quadratic	Quadratic	Linear	Linear
ESL	0.50	0.50	0.50	0.50	Quadratic	Linear	Linear	Linear
GBP	0.50	0.50	0.50	0.50	Quadratic	Linear	Linear	Linear
LAC	0.50	0.50	0.50	0.50	Quadratic	Quadratic	Linear	Linear
LEV	5.00	5.00	5.00	5.00	Quadratic	Linear	Linear	Linear
LTG	0.50	1.00	1.00	2.50	Quadratic	Quadratic	Quadratic	Linear
OXC	0.05	0.05	0.05	1.00	Linear	Linear	Linear	Linear
PGR	0.50	0.50	0.50	0.50	Quadratic	Linear	Linear	Linear
PHT	0.50	1.00	2.50	10.00	Linear	Linear	Linear	Linear
p-HPPH	0.50	1.00	2.50	10.00	Linear	Linear	Linear	Linear
RTG	0.05	0.50	0.50	1.00	Quadratic	Linear	Linear	N/A
TIG	0.05	0.05	0.25	0.50	Quadratic	Linear	Linear	Linear
TPR	0.50	0.50	0.50	2.50	Linear	Linear	Linear	Linear
VIG	5.00	5.00	5.00	5.00	Quadratic	Linear	Linear	Linear
VPA	5.00	5.00	5.00	5.00	Linear	Linear	Linear	Linear
ZNS	0.50	1.00	2.50	5.00	Linear	Linear	Linear	Linear

Often, the mobile phase or a similar solution are used for reconstitution when using LC/MS/$(MS)^n$. This may not always be applicable in practice where drugs may not be soluble enough in the percentage of organic/aqueous phase used which can exhibit suppressing or enhancing effects on the analyte response (249). Interestingly, this study showed that mobile phase may not always be the most appropriate reconstitution solution. In the case of AEDs, responses were significantly higher when the supernatant was diluted with only water or 0.1% formic acid in water compared to the dilution with mobile phase for both basic and acidic drugs (Figure 2-25). Again, this study proved the fact the less salt or additives present in the mobile phase, the better the response (see 2.3.3.3). Furthermore, dilution with water or 0.1% formic acid in water improved the LOQ for some drugs like ZNS, PHT and its metabolite p-HPPH from 2.5 to 1 mg/L. Responses attained from using either water or 0.1% formic acid in water were almost comparable, hence, water was used as the reconstitution solution of choice.

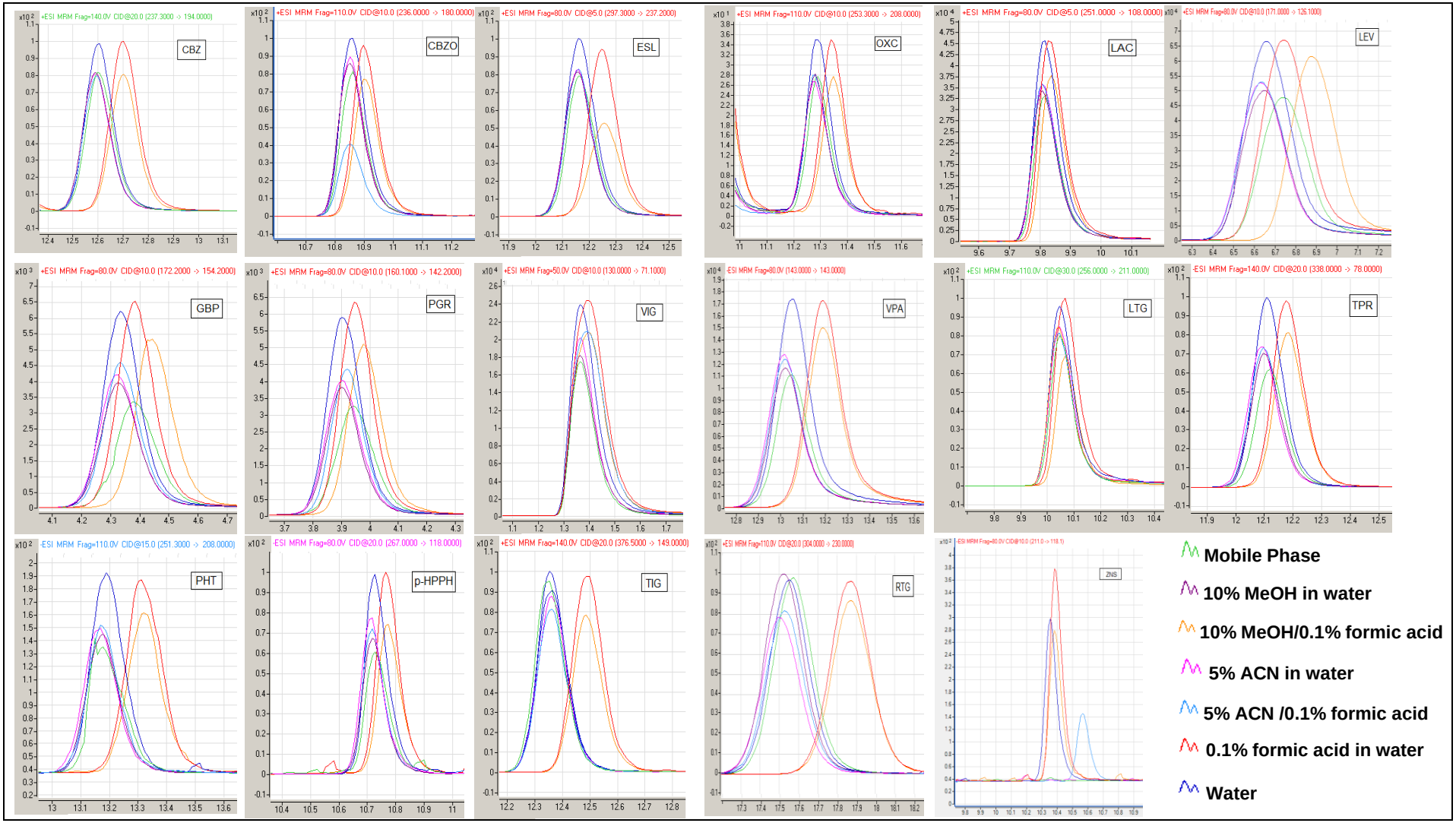


Figure 2-25: Effect of 7 Different Reconstitution Solution Compositions on AED Response.

2.3.8 Investigation Into Protein Precipitation Extraction Conditions

2.3.8.1 Effect of Extraction Solvent

A total of 7 different solvents combinations were assessed in triplicate. Figure 2-26 shows graphic representations of the recovery results for the 7 extraction methods. All solvent combinations used provided a recovery higher than 80% for all the AEDs except VIG (70%) and RTG (less than 50%). Although extraction yields were good with all solvents used, the methanol and acetonitrile extractions were much cleaner.

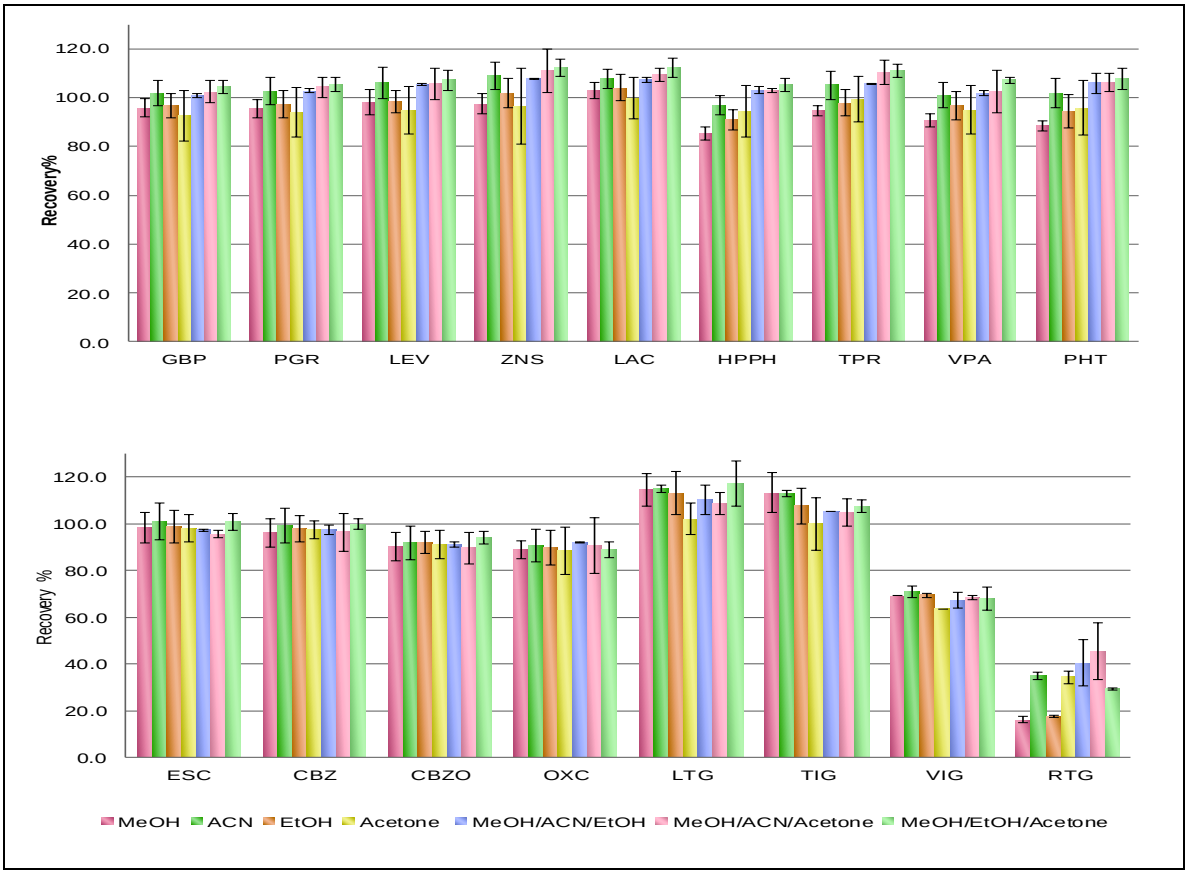


Figure 2-26: Comparison of AEDs Recoveries with 7 Different Solvent Combinations.

2.3.8.2 Effect of Centrifuge Duration and Speed

Centrifuge duration was assessed at 10, 15 and 20 minutes. Figure 2-27 shows that centrifuge duration does not have a significant effect on the extraction yield of these drugs, although CBZ and its derivatives (OXC, ESL and CBZO) showed a higher recovery by 10% after 10 minutes centrifuge.

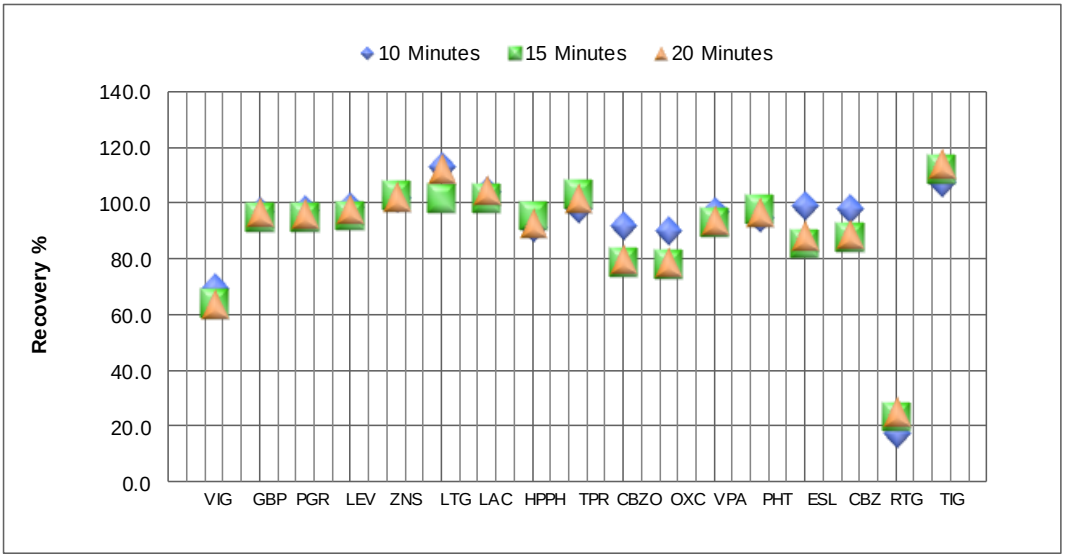


Figure 2-27: The Effect of Centrifuge Duration on Extraction Yield.

Figure 2-28 shows that increasing the centrifuge speed improved the extraction yield by 10-20% for all drugs. Although LTG and TIG showed a higher recovery (>100%) with 5000 rpm centrifuge speed which may be due to increased matrix effects, the standard deviation was lower and results were more robust when 10000 rpm used. Hence, a centrifuge time of 10 minutes at 10000 rpm was chosen for protein precipitation extraction of AEDs.

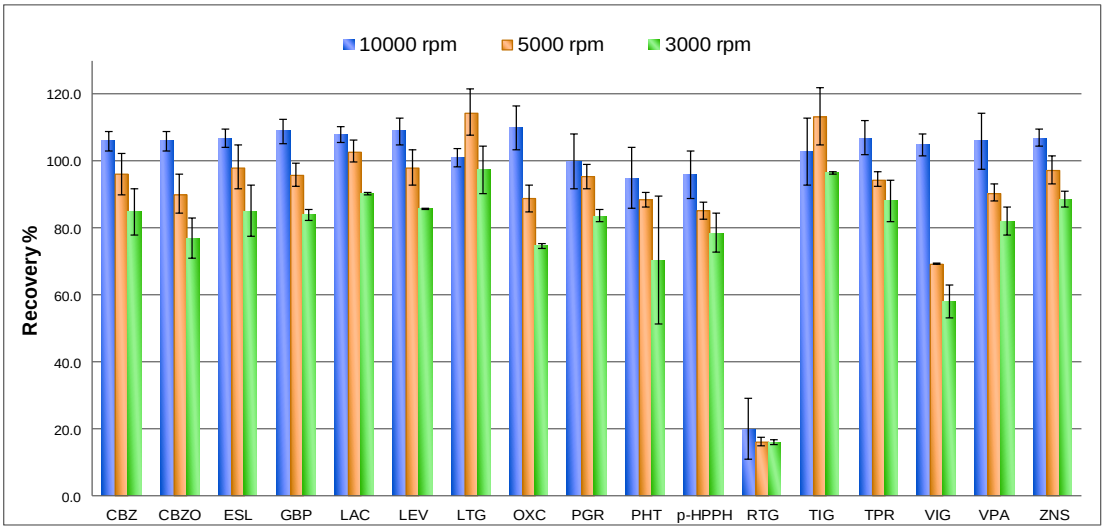


Figure 2-28: The Effect of Centrifuge Speed on Extraction Yield.

2.3.8.3 Effects of Water Haemolysis

For AEDs, water haemolysis (1:1 v/v) does not improve the extraction yield and the recovery was lower by 5-20% compared with non-haemolysed blood samples Figure 2-29. As a result, protein precipitation was applied without water haemolysis.

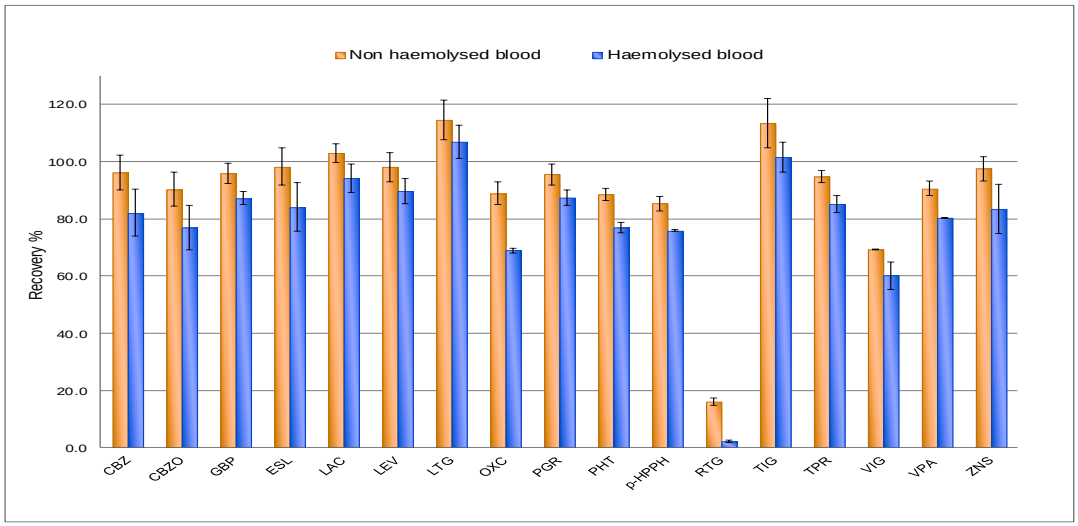


Figure 2-29: The Effect of Water Haemolysis Step of Blood Sample on Extraction Yield.

(100 µL Blood Sample was Diluted with 100 µL Water Before the Sample Undergo Protein Precipitation Extraction)

2.3.8.4 Matrix Effect Evaluation

Since methanol and acetonitrile extractions were found to provide higher recoveries with cleaner supernatants, the matrix effect was assessed for these two extraction methods. Figure 2-30 shows an acceptable matrix effect with both extractions (within ± 25%) except for RTG which shows a poor recovery and an unacceptable matrix effect. The matrix effect is slightly lower using ACN extraction but it exceeded the acceptable range with TIG (29%), while all AEDs showed acceptable values with MeOH extraction.

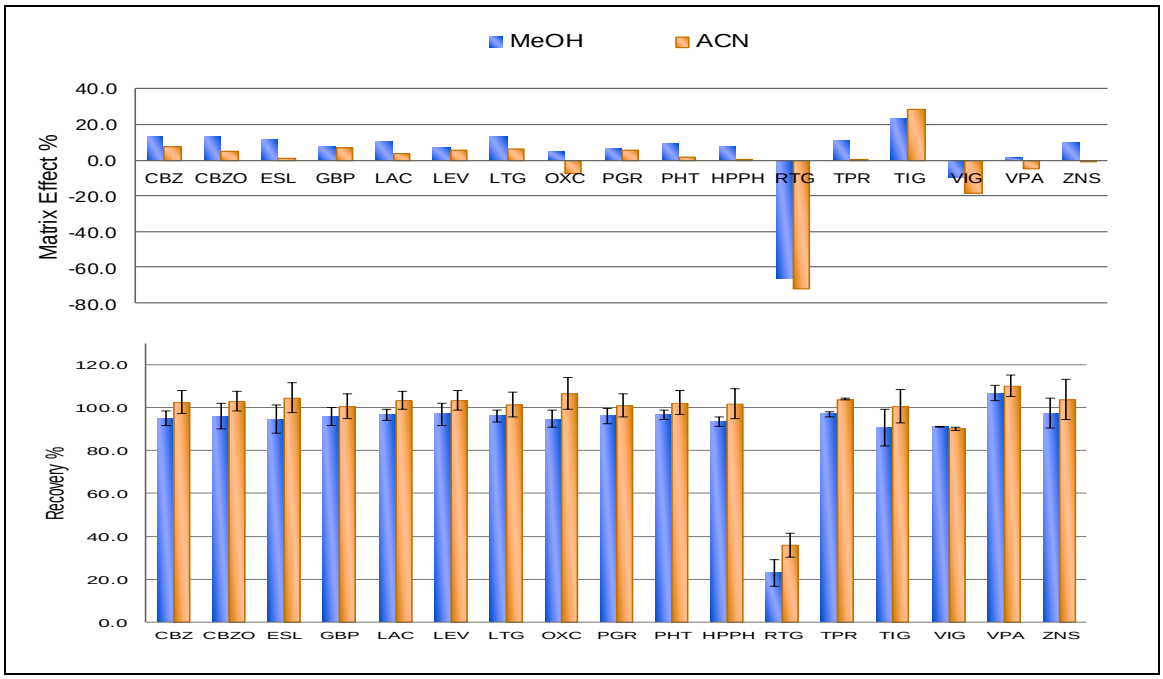


Figure 2-30: Comparison of the Matrix Effect and Recovery Yields for Two Different Protein Precipitation Methods Used Methanol and Acetonitrile as Solvents and Post Extraction Approach (Matuszewski Strategy).

Extraction recovery in Figure 2-30 was calculated using the Matuszewski strategy which uses the mean of the analyte peak area only whereas the recovery mentioned previously was calculated using analyte/internal standard peak area ratios. A comparison between recovery values obtained using both of these methods is illustrated in Figure 2-31. Both methods gave comparable results, although recovery calculation of vigabatrin was about 20% higher using the Matuszewski strategy which may due to eliminating the matrix effect in case of post extraction approach..

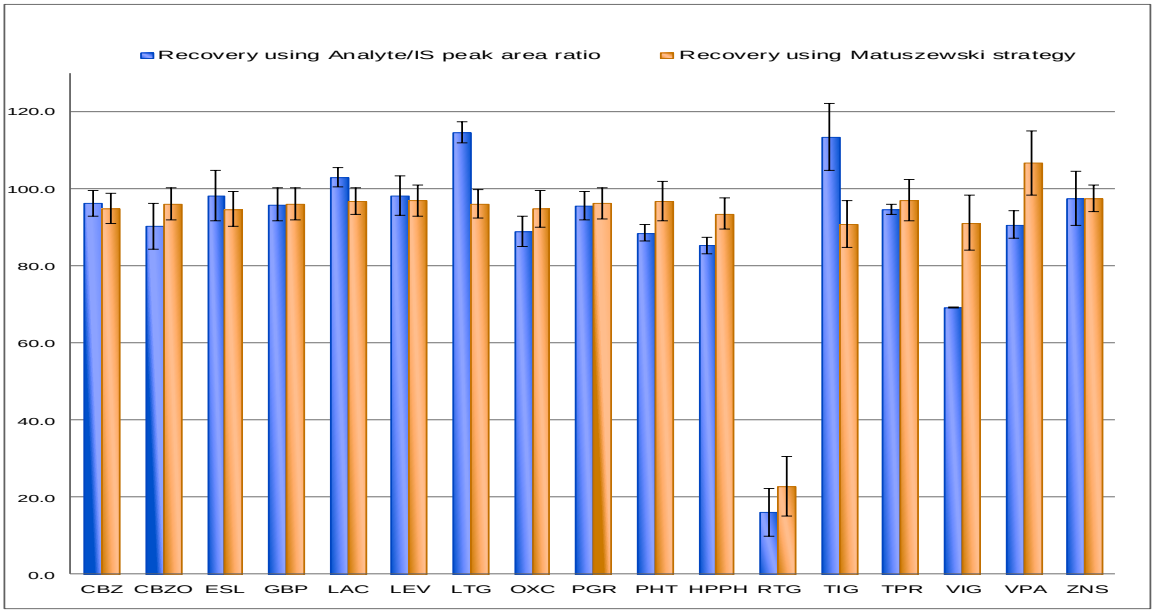


Figure 2-31: Comparison of Recovery Values Calculated Using Matuszewski Strategy Which Uses Peak Area Only and Recovery Values Calculated Using Analyte/IS Peak Area Ratio.

2.4 Conclusion

The separation of 15 AEDs, 2 metabolites and 3 internal standards was achieved using an Agilent LC/MS/MS triple quadruple coupled with a Gemini C18 column (150 mm x 2.0 mm, 5µm) and a C18 guard column (4.0 mm x 2.0 mm) maintained at 40 °C. Electrospray ionization was used and the MS operated in dynamic multiple reaction monitoring mode (DMRM) with ion mode switching. The optimal MS conditions were achieved using a nebulizer pressure of 15 psi, a capillary voltage of 4,000 V, nitrogen gas heated to 300 °C and delivered at 10 mL/min.

Gradient elution was employed using a mobile phase consisting of A: 2 mM ammonium acetate in water and B: 2 mM ammonium acetate in methanol at a flow rate of 0.3 mL/min. The total run time was 17 min. The gradient mobile phase system started at 80:20 A/B increasing to 50:50 A/B within 2 min. This percentage was maintained for 6 min before

being increased to 10:90 A/B for 2 min. The percentage was finally decreased to 80:20 A/B for 7 min in order to condition the column before the next injection.

The complex mixture of AEDs used in this method required a very general method in order to attain high recoveries with all drugs of interest. Although Strata X cartridges are recommended for general SPE extraction they did not achieve the optimum results in the case of AEDs.

Protein precipitation gave higher recoveries with all AEDs (>70%) except RTG (<50%). Although, it is not a highly clean method, the purity of the samples was sufficient to be injected into LC/MS/MS. On the other hand, it was simple, fast, time saving and little solvent was required.

It is important as part of any method development to evaluate the effect of reconstitution volume and composition due to its significant effect on linearity, LOQ and calibration model. This study showed that reconstitution with mobile phase may not give the optimum results and sensitivity can be improved by investigation of different solution compositions. Although most of the solutions used were similar to the mobile phase composition, a slight modification on the percentage of the reconstitution composition could result in a considerable change in the analyte response which in turn improved the sensitivity of the analysis.

Hence, it was decided to use protein precipitation as the extraction of choice for the analysis of the 17 AEDs and a further investigation into the factors that might affect protein precipitation extraction was carried out.

The two precipitants found to yield highest recoveries with lowest standard variations were methanol and acetonitrile. Extraction with methanol centrifuged at 10000 rpm for 10 minutes without water haemolysis was considered as the method of choice because it was cheaper and simpler.

To sum up, the extraction of 15 AEDs and 2 metabolites was carried out using protein precipitation extraction as follows; a 100 µL of spiked blood was transferred to a 2-mL micro-centrifuge tube, 100 µL of internal standard solution and 300 µL of MeOH were added, vortex mixed for 10 seconds and centrifuged for 10 minutes at 10000 rpm. A 200 µL aliquot of the supernatant was transferred to a LC vial and diluted to 1.5 mL with

deionized water. 10 µL were injected into the LC/MS/MS. This extraction procedure was used to validate the method quantitively in the following chapter.

3 Method Validation of 17 AEDs in Whole Blood Using LC/MS/MS

3.1 Introduction

Before a new analytical method can be employed for the quantitative determination of drugs and their metabolites in biological samples, it has to be validated to fit the purpose of use. Method validation is a prerequisite to prove that an accurate, precise and rugged method has been developed to yield reliable results which can be satisfactorily interpreted (250).

Many protocols regarding method validation process have been published in the literature by different organisations to ensure high quality and reliable data such as FDA Guidance for industry-bioanalytical method validation (2001) (251), SOFT/AAFS forensic toxicology laboratory guidelines (2006) (252), the United Kingdom and Ireland association of forensic toxicologists forensic toxicology laboratory guidelines (UKIAFT 2010) (253) and the standard practices for method validation in forensic toxicology which was published by the Scientific Working Group for Forensic Toxicology (SWGTOX) in May 2013 (242). All these protocols and many others aim to improve method development and validation procedure, to implement good laboratory practice which in turn gives the most reliable results.

Method validation criteria vary depending on whether the method will be used for qualitative or quantitative analysis. In case of qualitative method, only few parameters need to be investigated; the limit of detection, specificity/selectivity, carryover and stability of analyte in given matrices. More parameters are required in case of quantitive analysis as drug concentration needs to be reported. These parameters include linearity, accuracy in addition to limit of detection, limit of quantification, specificity/selectivity, carryover and stability. Furthermore, a matrix effect assessment should be carried out in case of LC/MS/MS analysis.

The aim of this project was to quantitively validate the method developed in chapter 2 according to the standard practices for method validation in forensic toxicology (SWGTOX, May 2013) for whole blood as most up to date guidance available at the time of this project (242).

3.2 Materials and Methods

3.2.1 Materials

These were the same as those described in 2.2.1

3.2.2 Calibrators, Internal Standards and QCs Preparation

See 2.2.2.2 and 2.2.2.3 for calibrators, QCs and internal standards solutions preparation. All QCs and stock solutions were stored at -20°C and working solutions were stored at 4°C. Calibration curves were extracted in duplicate and quality controls in triplicate by adding the following volumes to 2 mL Eppendorf tube:

Calibrator: 100µL of whole blood, 100µL of calibration standard, 100 µL of internal standard solution and 200 µL of methanol (methanol total volume 400 µL).

QC: 100 µL of internal standard solution and 300 µL of methanol (methanol total volume 400 µL).

The calibrators and QCs were vortex mixed for 30 seconds and centrifuged for 10 minutes at 10000 rpm. An aliquot of 200 µL of the supernatant was transferred to an LC vial and diluted to 1.5 mL with deionized water. A 5 µL aliquot of the diluted supernatant was injected and analysed by LC/MS/MS.

3.2.3 Instrumentation

An Agilent LC/MS/MS triple quadruple G620A mass spectrometer equipped with Agilent 1200 series auto sampler, quaternary pump SL with degasser and thermostatted column compartment was used. Electrospray ionization (ESI) was used and the MS operated in dynamic multiple reaction monitoring mode (DMRM) with ion mode switching. The optimal conditions were achieved using a nebulizer pressure at 15 psi, a capillary voltage of 4000 V, nitrogen gas heated to 300 °C and delivered at 10 mL/min. The column used was a Phenomenex Gemini C18 (150 mm x 2.0 mm, 5µm) coupled with a C18 guard column (4 x 2.1 mm). The column temperature was maintained at 40 °C. Gradient elution was employed using a mobile phase consisting of A: 2 mM ammonium acetate in water and B: 2mM ammonium acetate in methanol at a flow rate of 0.3 mL/min. The total run time was 17 minutes. The gradient mobile phase system started at 80:20 A/B increasing to

50:50 A/B within 2 minutes. This percentage was maintained for 6 minutes before being increased to 10:90 A/B for 2 minutes. The percentage was finally decreased to 80:20 A/B for 17 minutes in order to condition the column before the next injection. Data analysis was performed using Agilent Mass-Hunter Workstation (version: B.01.05).

3.2.4 Selectivity and Specificity

Selectivity represents the extent to which an analytical method can be used to distinguish the target analyte in a complex matrix without any interference from other components of similar behaviour (such as metabolites, impurities, degradation) whereas specificity is defined as the ultimate selectivity where interference possibility is 0 %. Due to the confusion of using both terms, it has been recommended to use the term "Selectivity" and discourage the term "Specificity" because a method can be either specific or not and it is rare to have a very specific method for an analyte without any interference (254, 255).

Hence, a selectivity study was carried out to determine if there were any potential interferences from matrix components (endogenous) by comparing the chromatograms of 11 different blank sources (donors) with those of corresponding standards spiked with 17 AEDs at a concentration of 10 mg/L. Specificity was assessed by spiking drug-free matrix with each AED and internal standard individually at a concentration of 10 mg/L to evaluate the extent to which the method is selective for each AED and whether there is any interference among the AEDs of interest or their internal standards (exogenous) which might affect the quantitation results at a later stage.

3.2.5 Limits of Detection and Limits of Quantification

Instrument Limits of Detection LOD is considered the lowest concentration that gives a reproducible instrument response and which also can be distinguished from the matrix background noise with signal to noise ratio (S/N) $\geq$ 3 using non-extracted standards. Assay LOD is considered the lowest concentration that gives a reproducible instrument response with signal to noise ratio (S/N) $\geq$ 3 as well but using extracted standards, in other word, assay LOD is in the presence of the matrix effect (242).

The lower limit of quantification (LLOQ) is considered the lowest concentration that gives a reproducible instrument response with a relative standard deviation (RSD or %CV) less than 20 % and S/N $\geq$ 10. The limit of quantification (LOQ) was determined on the basis of the clinical need and should have a %CV less than 20% and S/N $\geq$ 10.

Instrument LOD is determined using decreasing concentrations of non-extracted drugs standard solutions within the expected range of the LODs; 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 mg/L for levetiracetam, vigabatrin and valproic acid (group 3); 0.005, 0.01, 0.05, 0.1, 0.25, 0.5 and 1 mg/L for other AEDs . The samples were analysed in duplicate for three separate runs.

Assay LODs, LLOQs and LOQs were determined using drug free blood spiked with the same decreasing concentrations of AEDs used to determine the instrument LODs and also analysed in duplicate for three separate runs using three different sources (donors) of blank blood. Mass-Hunter Workstation program was used to calculate the S/N ratio.

3.2.6 Linearity

One of the main purposes of developing this method was to evaluate the linearity with a wide range covering therapeutic and toxic concentrations. Calibration curves with a wide range would be important in toxic cases where laboratories are often required to repeat the analysis using a sample dilution procedure to obtain an accurate concentration. This is because the calculated results are higher than the upper limit of quantification (ULOQ) of the method. Hence, linearity was assessed by analysing five separate calibration curves prepared by spiking blank blood with AEDs working solutions at 8 concentrations ranging from 5-300 mg/L for levetiracetam, vigabatrin and valproic acid; 0.05-10 mg/L for oxcarbazepine, retigabine and tigabine and; 0.5-50 mg/L for carbamazepine, carbamazepine epoxide, eslicarbazepine acetate, gabapentin, pregabalin, lacosamide and topiramate; 1-50 mg/L for lamotrigine, phenytoin, p-HPPH and zonisamide. All calibrations were prepared freshly in duplicate over 5 different days. Calibration curves were generated by plotting the peak area ratio versus the spiked analyte concentrations using the simplest least-squares linear regression model and Agilent Mass-Hunter Workstation-Quantitive software. A blank blood extract containing internal standard was run with each batch but not included in the calibration curve. The correlation coefficient (R^2) was calculated. The R^2 values should be greater than 0.99.

3.2.7 Bias and Precision

Bias (accuracy) describes the closeness of mean concentration obtained by the method to the true concentration of the analyte. Precision is defined as the closeness of agreement

(degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous samples under the prescribed conditions (251).

Bias (accuracy) and precision were assessed by analysing replicates of spiked controls at 3 different concentrations (low, medium and high). A calibration curve was prepared with each batch of QCs using the optimised method to calculate the concentrations. Intra-day (within) precision and bias were calculated from 6 replicates per QC in one batch. Inter-day precision and bias were determined over 5 different runs. Their values were calculated using the following equations (242):

$$Mean\ measured\ concentration\ \ \bar{X} = {\sum x_i}/{n}$$

$$Standard\ deviation\ (SD) = \sqrt{{\sum (x - \bar{x})^2}/{n-1}}$$

$$Bias\ (\%) = \frac{\bar{X}}{X} \times 100\ \ (X\text{: } nominal\ concentration)$$

$$Intra-day\ run\ precision\ (\%CV) = \frac{SD\ for\ single\ run\ of\ samples}{\bar{X}\ for\ single\ run\ of\ samples} \times 100$$

$$Inter-day\ run\ (\%CV) = \frac{SD\ for\ each\ concentration\ over\ 5\ runs}{\bar{X}\ for\ each\ concentration} \times 100$$

Bias was expressed as a percentage of the nominal concentration. The mean value should not deviate by more than 15% from the true value (±15%). Precision was established by the percentage of the co-efficient of variation (%CV). The results are considered acceptable if %CV is less than 15%.

3.2.8 Recoveries and Matrix Effects

Recoveries and matrix effects were evaluated for all the drugs and the internal standards using the post-extraction addition approach (see 2.2.12.4). Eleven different blank blood sources (donors) were spiked with either low or high QC and analysed in triplicate. Matrix effect was represented by the Matrix Factor (MF). Recovery was calculated by dividing the mean peak area of extracted standards (pre-extraction spike) by the mean peak area of the

extracted pooled matrix spiked with standards after extraction at the same concentration (post-extraction spike). Matrix effect is considered acceptable if the Matrix Factor was within a range of 1± 0.25.

3.2.9 Carryover

Carryover was tested by injecting three blank controls after two injections of a high concentration (QC4) double the upper limit of quantification in the calibration curve (500 mg/L for levetiracetam, vigabatrin and valproic acid; 20 mg/L for oxcarbazepine, retigabine and tigabine and 100 mg/L for the rest of the AEDs). Carryover was evaluated by examining the chromatograms visually.

3.2.10 Stability

As part of method validation requirements, drug stability has to be investigated under preparation and analysis conditions in order to ensure accurate quantitative results. Storage conditions evaluated were:

a) Bench top stability at room temperature (18±2 °C) for 24 hours.

b) In process (auto sampler) stability (~ 20ºC) for 24 hours.

c) Stability after 3 freeze-thaw cycles at -20±2 ºC. Samples were left to defrost at room temperature and analysed in triplicate after each freeze-thaw cycle.

Drug-free blood was spiked with the 17 AEDs at 2 concentrations (low and high). Freshly spiked samples were initially extracted and analysed in triplicate to establish time zero concentrations. For each concentration, 100 µL aliquots were placed into labelled 1.5 mL Eppendorf tubes and stored under previous conditions until analysis. Subsequently, samples were extracted and processed in triplicate along with freshly spiked calibration standards and analysed using the regression equation obtained. The calculated concentrations were compared with the average time zero concentrations. Recovery was calculated by dividing the calculated concentration after 24 hours by the concentration at time zero and multiplied by 100. The analyte was considered stable if the concentration was within ± 10% of the time zero concentration.

3.2.11 Dilution Integration

Since many forensic cases may involve overdose or lethal concentrations of drugs (higher than ULOQ), dilution integrity was evaluated. Dilution integrity was assessed by spiking blank whole blood with a concentration (QC4) higher than the ULOQ (500 mg/L for levetiracetam, vigabatrin and valproic acid; 20 mg/L for oxcarbazepine, retigabine and tigabine; and 100 mg/L for the rest AEDs) and diluting this with blank blood at dilution factors of 1:2, 1:5 and 1:10. Four replicates per dilution factor were prepared and processed along with freshly spiked calibration standards and analysed by back calculation using the calculated regression equation. Dilution of samples should not affect the accuracy and precision. The integrity of the samples was considered to be maintained if the mean concentration obtained was within ± 15% of nominal values and %CVs ≤ 15% at all diluted concentrations.

3.3 Results and Discussion

3.3.1 Selectivity and Specificity

It was obvious that there were no endogenous interferences as illustrated in Figure 3-1 (a-e). None of the AEDs or the internal standards showed any interference at the peak area of the other drugs included in the method. The method was selective and specific for AEDs of interest and their internal standards. The gabapentin chromatogram exhibited a peak at a retention time of 2.4 minutes which does not interfere with gabapentin which has a retention time of 3.3 minutes and these are completely resolved. The contamination source of this peak could not be determined. It may have come from plasticisers and plastic materials such as phthalate esters and polypropylene glycols which have been reported as interferents in methods using positive ionisation mode in mass spectrometry or it could be a matrix component such as phospholipids (256). Plasticisers and other plastic materials may be present as a result of using plastic consumables during the sample preparation process or may be released from the plastic bank blood used by the hospital (257).

(a)

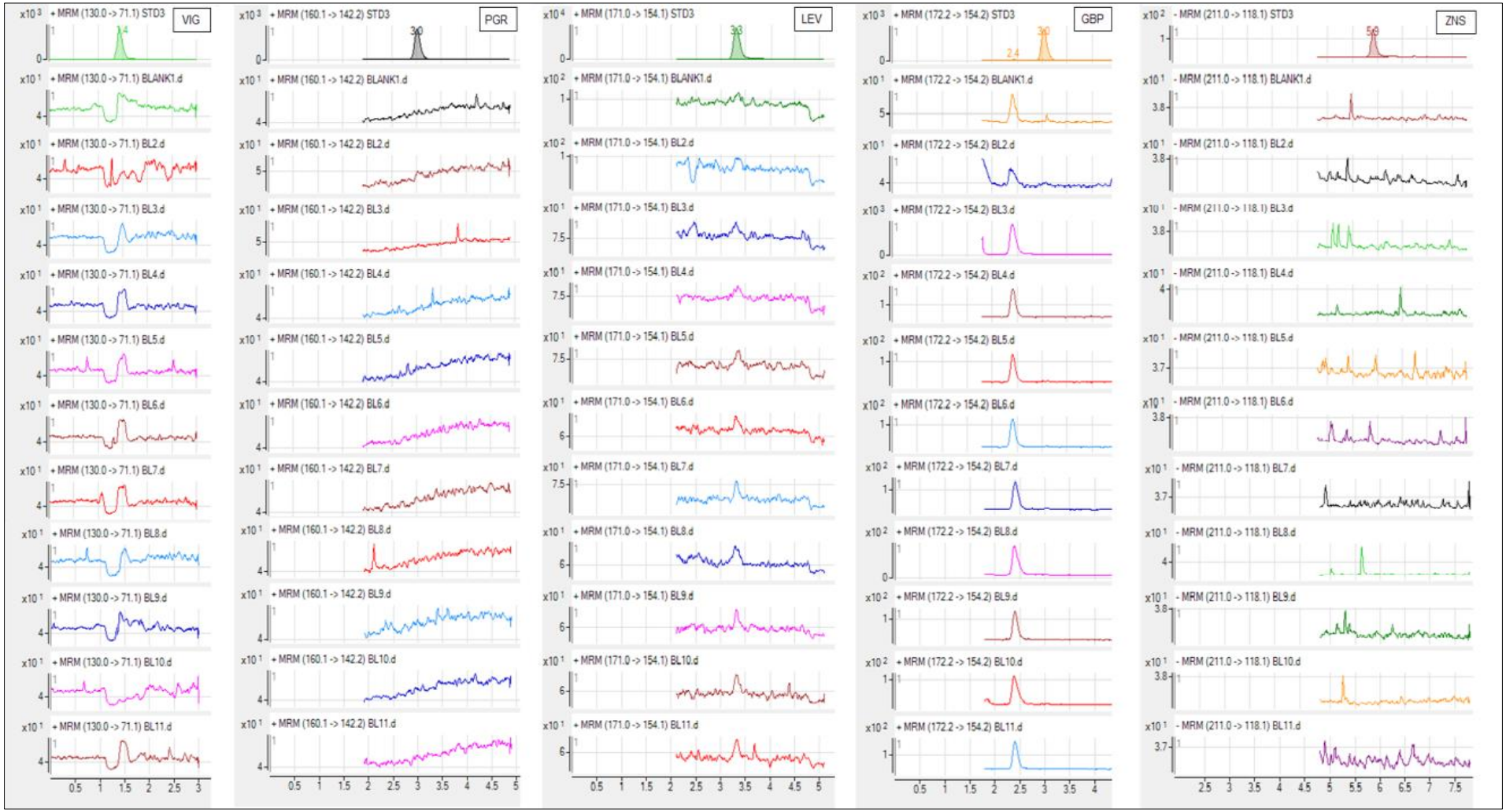


Figure 3-1 (a-e): The Chromatograms of 11 Drug Free Whole Blood Sources Compared to AED Standard Mix at 10 mg/L.

(b)

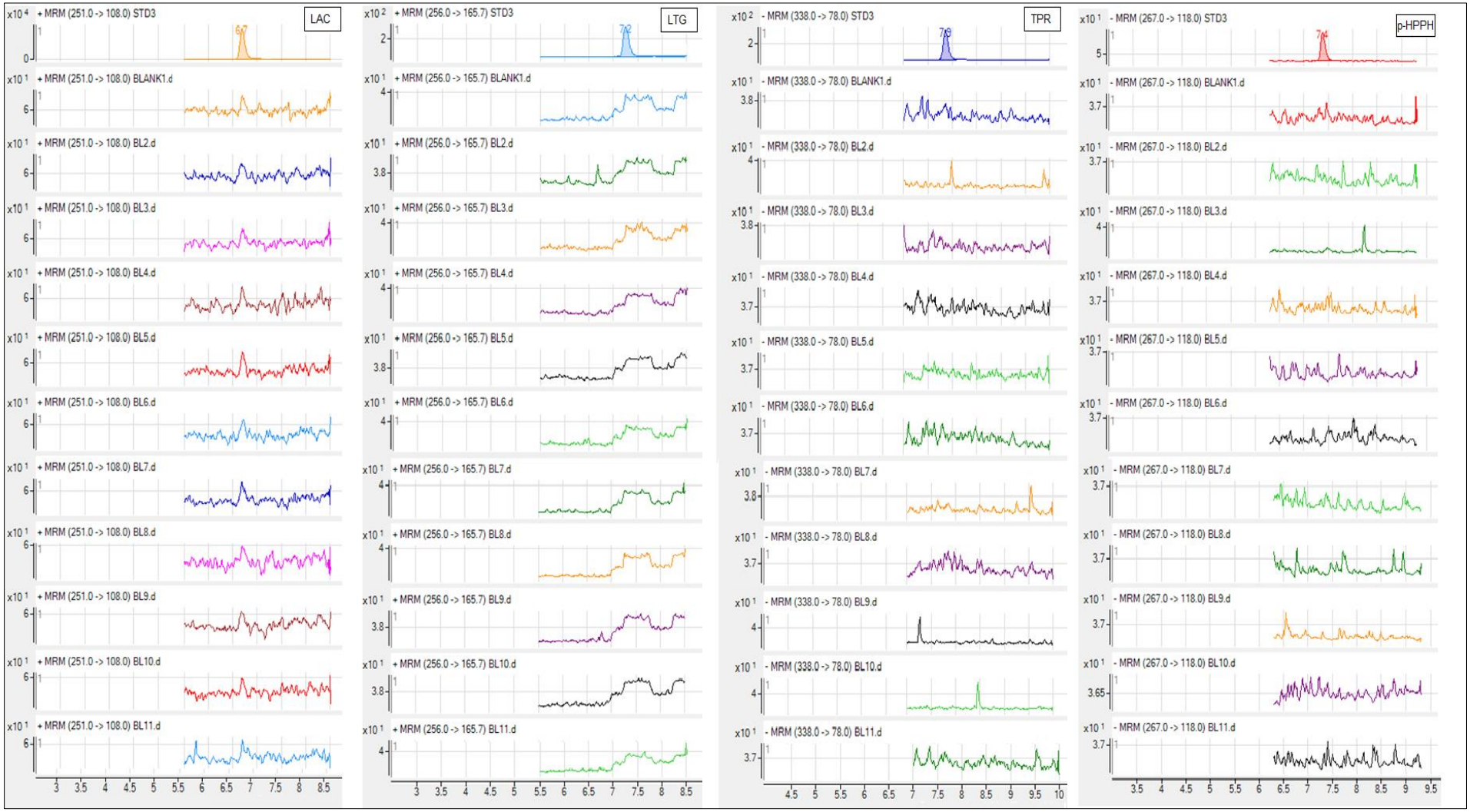


Figure 3-1(a-e): The Chromatograms of 11 Drug Free Whole Blood Sources Compared to AED Standard Mix at 10 mg/L (Continued…).

(c)

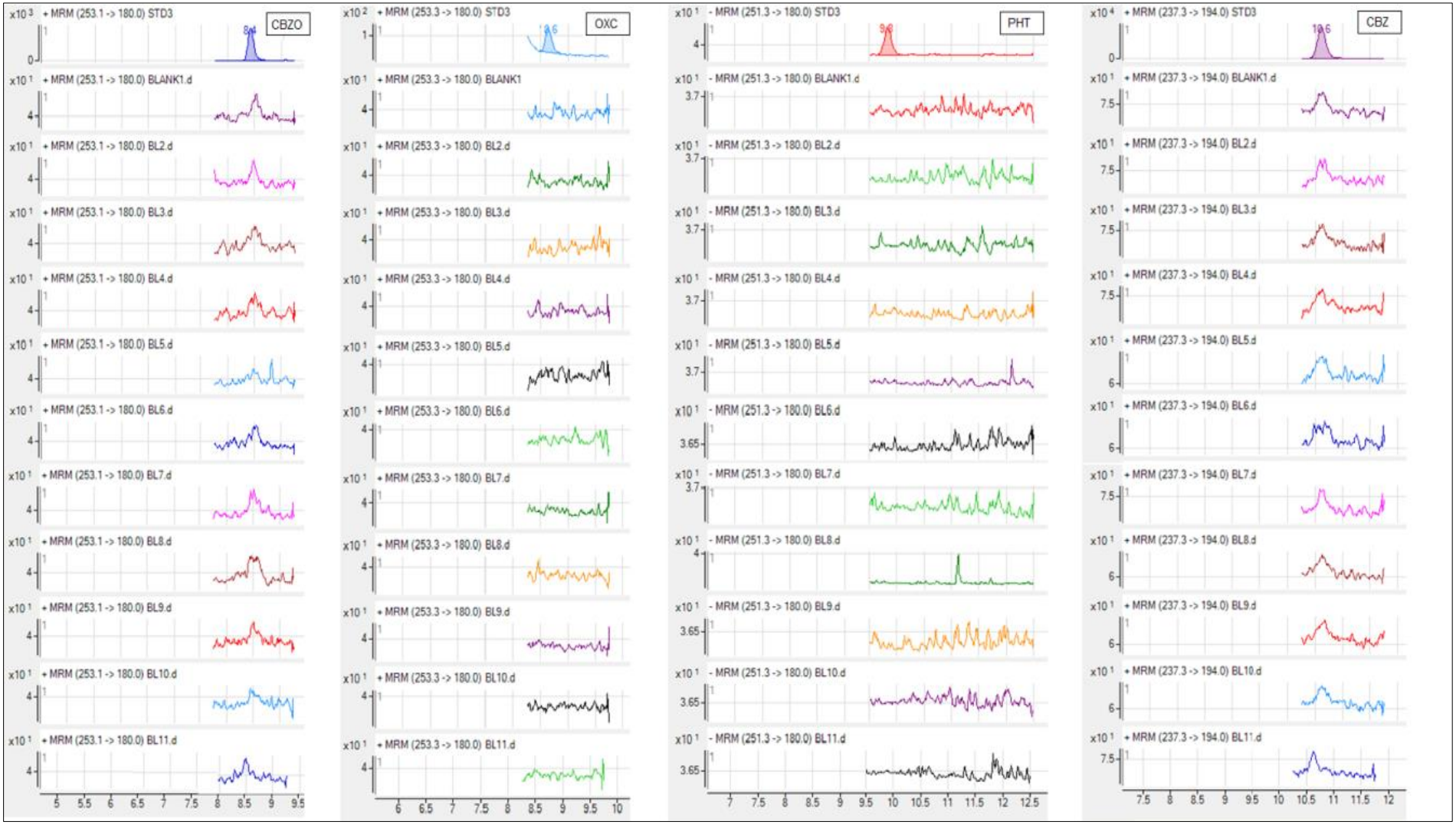


Figure 3-1(a-e): The Chromatograms of 11 Drug Free Whole Blood Sources Compared to AED Standard Mix at 10 mg/L (Continued…).

(d)

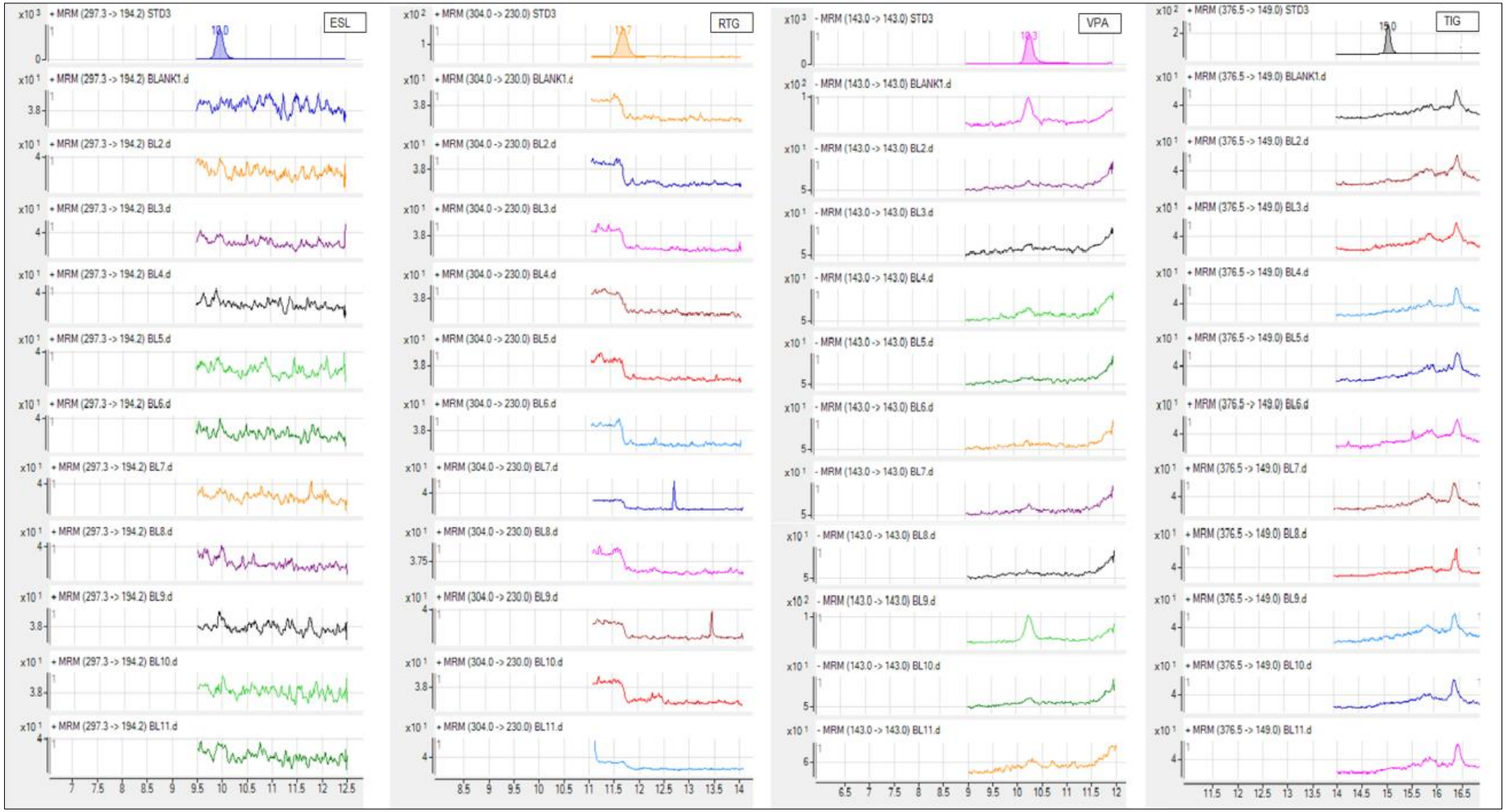


Figure 3-1(a-e): The Chromatograms of 11 Drug Free Whole Blood Sources Compared to AED Standard Mix at 10 mg/L (Continued…).

(e)

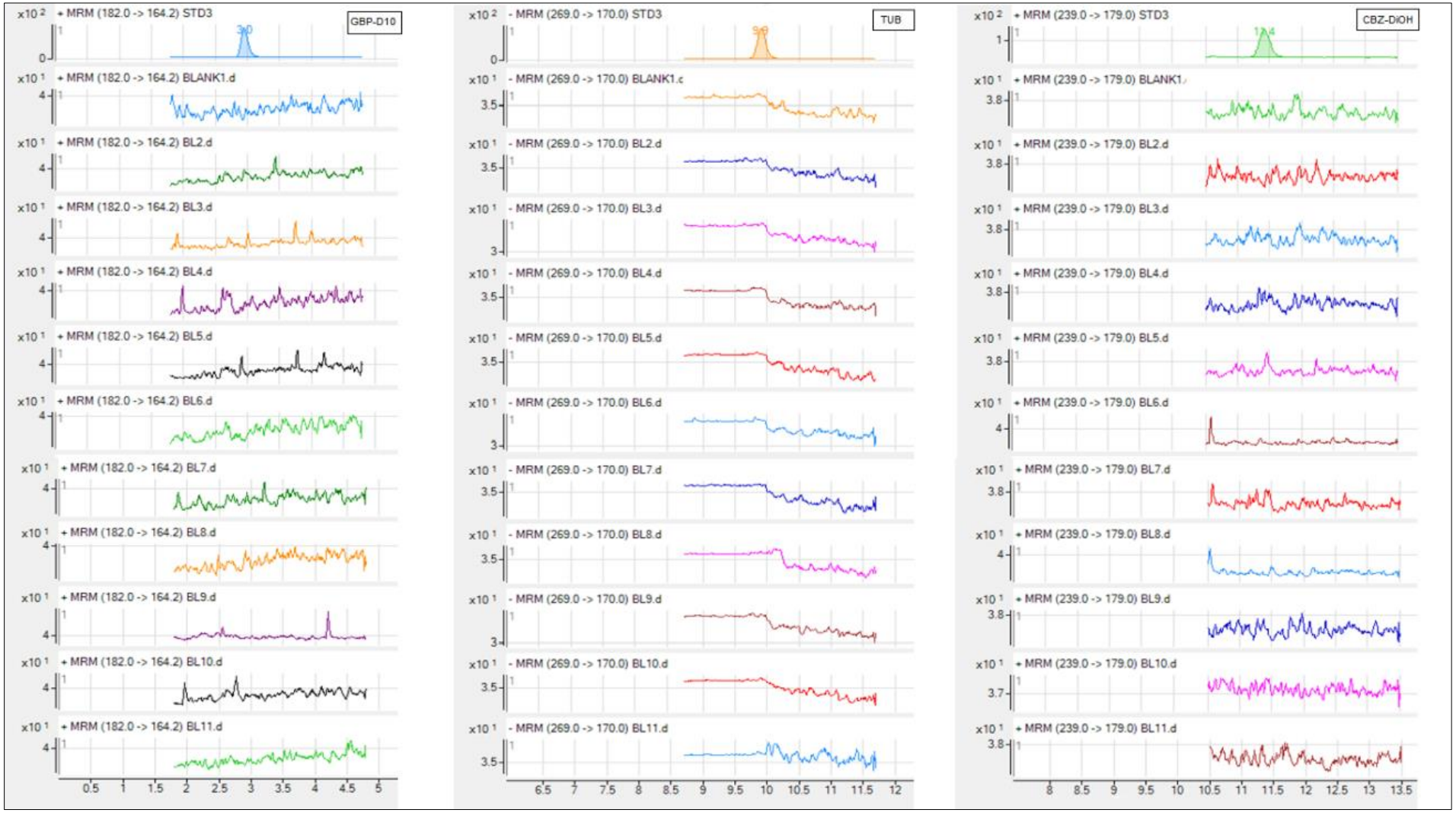


Figure 3-1 (a-e): The Chromatograms of 11 Drug Free Whole Blood Sources Compared to AED Standard Mix at 10 mg/L (Continued…).

3.3.2 LOD and LOQ

Instrument and Assay LOD, LLOQ and LOQ results of 17 AEDs are presented in Table 3-1. Instrument and assay LOD results were almost identical for most AEDs except valproic acid, vigabatrin, eslicarbazepine acetate and carbamazepine which showed lower instrument LODs (1 and 0.1 mg/L respectively) compared to their assay LOD (2.5 and 0.25 mg/L respectively) which may be due to matrix effects. The LOQ in the majority of cases was chosen to be the LLOQ. Only for 6 out of 17 drugs was it chosen to be higher than the LLOQ as the level of sensitivity achieved was not required.

Table 3-1: Instrument and Assay LOD, LLOQ and LOQ of 17 AEDs in Whole Blood.

AEDs	Instrument LOD	Assay LOD	LLOQ	LOQ
CBZ	0.1	0.25	0.5	0.5
CBZO	0.05	0.05	0.25	0.5
ESL	0.1	0.25	0.5	0.5
GBP	0.1	0.1	0.25	0.5
LAC	0.05	0.05	0.25	0.5
LEV	0.1	0.1	0.5	5.0
LTG	0.25	0.25	0.5	0.5
OXC	0.025	0.025	0.05	0.05
PGR	0.25	0.25	0.5	0.5
PHT	0.5	0.5	1.0	1.0
p-HPPH	0.5	0.5	1.0	1.0
RTG	0.025	0.025	0.05	0.05
TIG	0.01	0.01	0.025	0.05
TPR	0.25	0.25	0.5	0.5
VIG	0.1	0.5	1.0	5.0
VPA	1	2.5	5.0	5.0
ZNS	0.5	0.5	1.0	1.0

3.3.3 Linearity

The calibration curves were linear over the wide range of concentrations tested with a R^2 greater than 0.998. Therapeutic levels, calibration model, internal standards, and linearity represented by R^2 are summarized in Table 3-2.

Using a linear regression equation weighted 1/X (X represents concentration); all the calibration lines passed all the acceptance criteria except lamotrigine and retigabine. With lamotrigine, quadratic regression was used for curve fitting with 1/x as the weighting factor

and R^2 was greater than 0.996 in the 5 validation batches with good accuracy and precision results. Although a quadratic curve is not preferred in routine work, it has been reported to have been used with lamotrigine in the literature (258, 259) because lamotrigine did not show a good linearity at concentrations higher than 10 mg/L. However, the lamotrigine therapeutic range can reach 19 mg/L in cases of chronic treatment. This means more dilution steps and laboratory work, more consumables and time.

Table 3-2: Therapeutic Concentrations, Calibration Model and Linearity of 17 AEDs in Whole Blood.

AEDs	Therapeutic level (mg/L)	Calibration Range (mg/L)	Calibration Model	Internal Standard	Whole blood R^2 (*n=5*)
CBZ	1.7-15	0.5-50	Linear	CBZ-DiOH	0.9997
CBZO	0.5-2.0	0.5-50	Linear	CBZ-DiOH	0.999
ESL	10.0-26.0	0.5-50	Linear	CBZ-DiOH	0.9995
GBP	5.0-9.0	0.5-50	Linear	GBP-D_{10}	0.999
LAC	2.5-14.0	0.5-50	Linear	GBP-D_{10}	0.998
LEV	10.0-40.0	5.0-300	Linear	GBP-D_{10}	0.998
LTG	2.3-5.6	1.0-50	Quadratic	GBP-D_{10}	0.996
OXC	0.05-1.2	0.05-10	Linear	CBZ-DiOH	0.998
PGR	1.0-5.0	0.5-50	Linear	GBP-D_{10}	0.999
PHT	7.0-20.0	1.0-50	Linear	TUB	0.999
p-HPPH	1.0-40.0	1.0-50	Linear	TUB	0.999
RTG	0.51-1.85	0.05-10	n/a	CBZ-DiOH	n/a
TIG	0.03-1	0.05-10	Linear	CBZ-DiOH	0.999
TPR	2.4-27	0.5-50	Linear	TUB	0.9997
VIG	18-77	5.0-300	Linear	GBP-D_{10}	0.998
VPA	50-100	5.0-300	Linear	TUB	0.9995
ZNS	1.0-50	1.0-50	Linear	TUB	0.999

Regarding retigabine, this drug did not give a consistent response during method development and validation. It is the last to elute at 15 minutes whereas the method time is 17 minutes. It was noticeable that any slight change in column conditions or gradient program would give poor reproducibility. The reason behind this response fluctuation was investigated by checking the gradient system, the mobile phase flow rate and column conditions in addition to the LC binary pump. There was a fungi growing issue in the

mobile phase due to an unsuitable water source which increased the pump pressure. It was thought that this problem affected retigabine elution because it elutes at the end of the run. However, after trying to adjust all these parameters, none of them improved the peak response or consistency. Hence, the retigabine calibration curve and validation results were not acceptable. As a result, retigabine analysis was considered qualitative only in this method. Linearity graphs generated using Mass-Hunter quantitative program are illustrated in Figure 3-2.

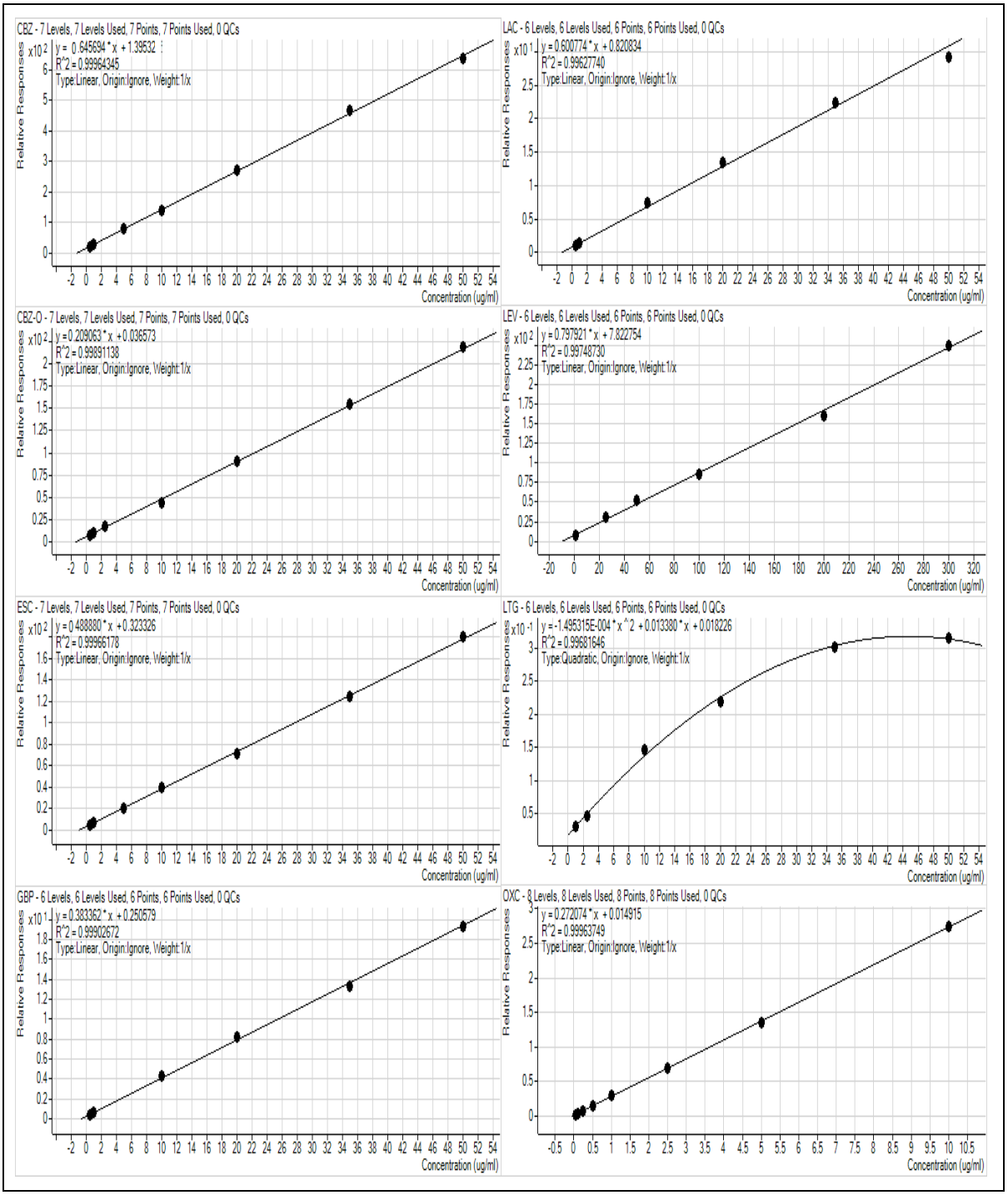


Figure 3-2: Linearity Graphs of 17 AEDs Generated Using Mass Hunter Quantitative Analysis.

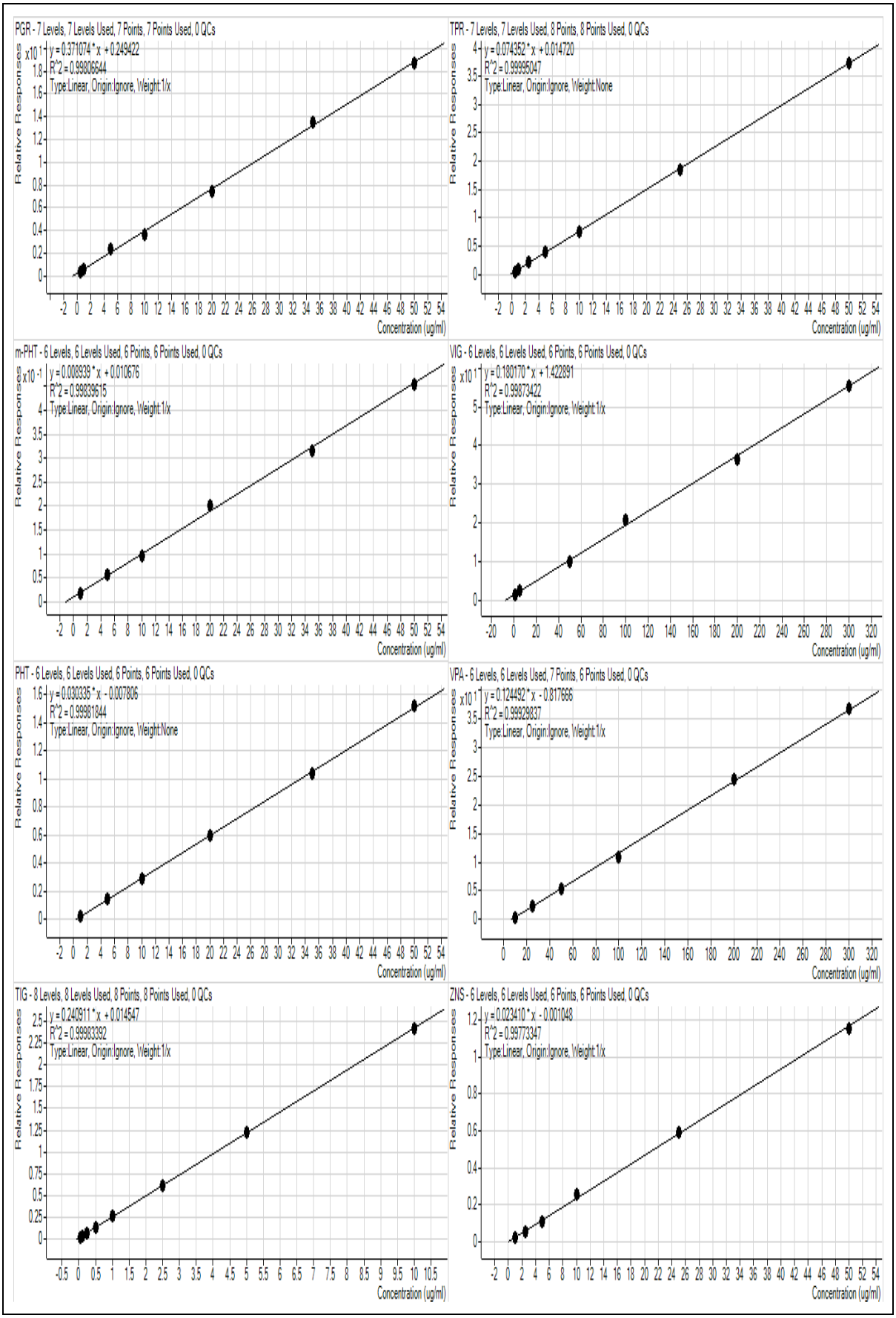


Figure 3-2: Linearity Graphs of 17 AEDs Generated Using Mass Hunter Quantitative Analysis (Continued…).

3.3.4 Bias and Precision

Accuracy results for 16 AEDs (except retigabine) are presented in Table 3-3. The accuracy values were within the acceptable range of ± 15% of the nominal concentrations. The intra-day accuracy was from 92.7 - 103.4%. The inter-day accuracy ranged from 92.7-108.7%. Both intra- and inter-day precision values were acceptable and less than 15%. Precision results of 16 AEDs (except retigabine) are showed in Table 3-4. The intra-day precision values were less than 9%. The inter-day precision values were less than 12.1%.

Table 3-3: Intra- and Inter-Day Accuracy Results of 16 AEDs.

AEDs	Accuracy *(n=30)*					
	Intra-day (%)			Inter-day (%)		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
CBZ	100.8	99.2	96.8	100.8	99.2	96.8
CBZO	95.9	101.5	102.5	95.8	101.5	102.5
ESL	97.5	101.9	98.8	97.5	101.9	98.8
GBP	99.9	102.7	100.6	102.4	102.7	100.6
LAC	92.7	102.7	99.7	92.7	102.7	99.7
LEV	95.1	99.3	101.3	95.1	99.3	101.3
LTG	101.9	95.9	100.3	101.9	103.3	100.3
OXC	102.7	97.5	103.1	102.7	97.5	103.1
PGR	98.3	101.4	102.1	98.3	101.4	102.1
PHT	101.7	98.2	101.1	101.7	98.2	101.1
p-HPPH	101.8	99.3	100.7	101.8	99.4	100.7
TIG	100.6	100.1	97.5	100.6	100.1	97.5
TPR	98.0	103.4	102.0	98.0	103.4	102.0
VIG	n/a	101.5	102.1	n/a	101.5	102.1
VPA	100.4	100.5	100.5	100.4	100.5	100.5
ZNS	94.3	98.2	100.3	94.3	98.2	100.3

Table 3-4: Intra- and Inter-Day Precision Results of 16 AEDs.

AEDs	Precision *(n=30)*					
	Intra-day (%)			Inter-day (%)		
	Low	Medium	High	Low	Medium	High
CBZ	2.6	2.8	1.6	9.2	3.8	4.6
CBZO	4.9	2.3	1.5	4.3	3.6	4.0
ESL	2.1	2.6	1.6	3.5	6.9	2.9
GBP	4.3	2.4	2.9	3.1	4.3	4.1
LAC	4.4	3.1	3.6	11.0	6.3	5.5
LEV	3.1	3.5	4.1	12.1	6.9	4.5
LTG	7.8	4.3	4.8	11.1	9.7	11.6
OXC	2.6	2.0	2.0	4.4	2.0	3.0
PGR	4.2	2.3	2.5	5.4	2.6	3.0
PHT	6.1	1.3	0.7	7.6	2.0	0.3
p-HPPH	8.4	2.8	2.2	8.1	8.5	4.3
TIG	5.7	2.4	2.0	6.8	4.8	3.9
TPR	2.7	1.7	1.7	5.5	4.0	4.7
VIG	n/a	4.3	2.8	n/a	5.8	4.0
VPA	1.2	0.7	0.4	4.8	0.3	0.4
ZNS	5.9	3.7	2.7	13.0	3.7	4.0

3.3.5 Matrix Effects and Recoveries

Table 3-5 shows the matrix factor and recovery results of 2 QCs (low and high) using 11 different sources (donors) of whole blood. Matrix factor values were within the acceptable range for all the drugs with standard deviations less than 25% except retigabine. Retigabine exhibited unacceptable matrix suppression with matrix factor (MF) of 0.33. Recovery was greater than 95% for all other AEDs at low, medium and high concentrations. It was unclear whether retigabine MF and recovery values resulted from actual matrix suppression or poor analysis response.

Table 3-5: Recovery and Matrix Factor Values for 17 AEDs Using Low and High QCs and 11 Different Whole Blood Sources (n=6 per QC per Matrix).

AEDs	QC1		QC2	
	Recovery (%)	*Matrix Factor*	*Recovery (%)*	*Matrix Factor*
CBZ	106±3.0	1.05±0.07	104±3.1	1.00±0.02
CBZO	105±3.2	1.05±0.05	104±1.6	1.01±0.02
ESL	107±2.7	1.07±0.05	106±1.9	1.04±0.04
GBP	109±3.6	0.99±0.08	106±2.6	1.01±0.03
LAC	108±2.5	1.06±0.07	107±2.3	1.00±0.02
LEV	109±4.0	1.08±0.06	109±5.9	0.97±0.02
LTG	101±22.7	1.14±0.22	98±12.6	1.08±0.16
OXC	110±6.5	1.00±0.07	108±7.3	0.91±0.03
PGR	100±8.2	1.11±0.08	107±4.8	1.04±0.03
PHT	95±9.2	1.09±0.08	106±7.1	0.99±0.05
p-HPPH	96±7.1	1.04±0.09	106±9.7	0.97±0.06
RTG	n/a	n/a	33±24.7	0.33±0.08
TIG	103±18.2	0.93±0.10	101±8.2	1.01±0.02
TPR	107±5.0	1.10±0.07	108±6.3	1.00±0.03
VIG	105±3.3	0.75±0.07	111±7.3	0.83±0.02
VPA	106±8.4	1.05±0.10	108±4.4	1.00±0.04
ZNS	107±2.5	1.05±0.08	105±4.7	0.99±0.02

3.3.6 Carryover

No carry over was observed in the blank samples after two consequent injections of the highest standard (QC4) as illustrated in Figure 3-3 using the total ion chromatogram graph and Figure 3-4 which shows the detailed chromatograms of 17 AEDs and the blank sample injected afterward in DMRM mode.

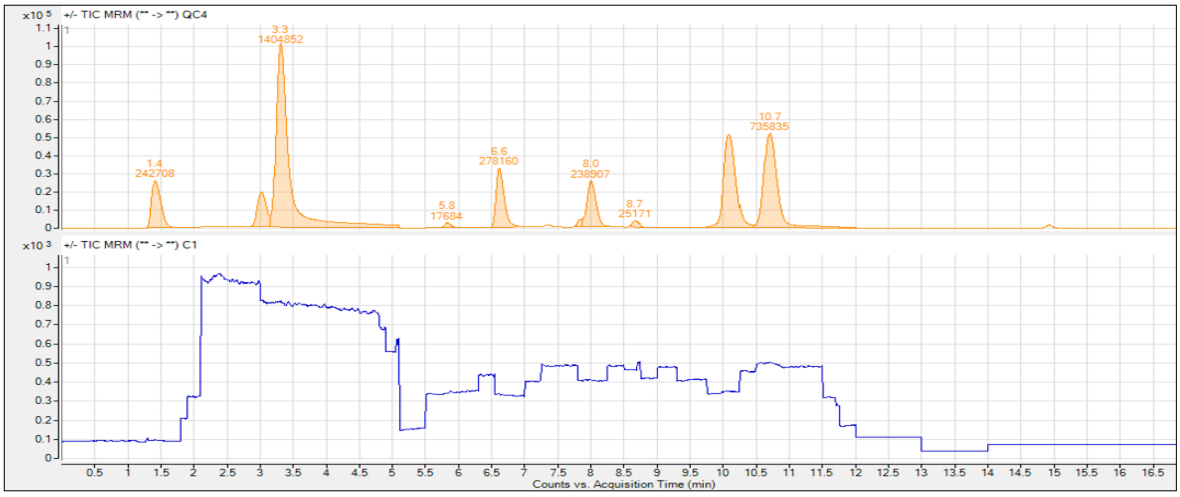


Figure 3-3: Carryover Results - Total Ion Chromatogram of High Concentration (QC4) Versus Blank Mobile Phase.

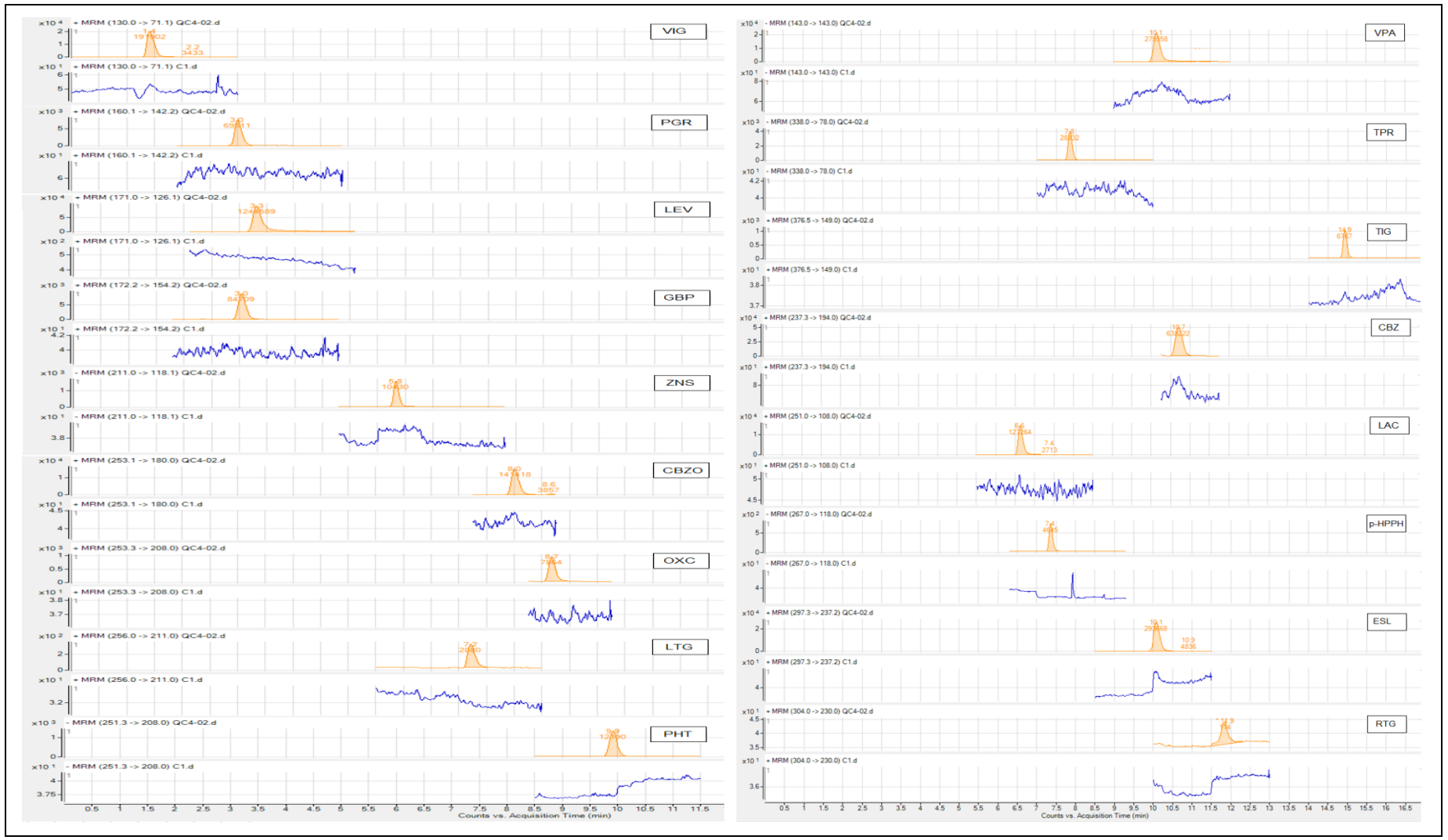


Figure 3-4: Carryover Results- DMRM Chromatogram of High Concentration (QC4) Versus Blank Mobile Phase.

3.3.7 Stability

Bench top stability: During the sample preparation process, the stability study showed that all the drugs were stable in the whole blood at room temperature (approximately 18ºC) for up to 24 hours except eslicarbazepine acetate, oxcarbazepine and retigabine which showed a loss of approximately 25 to 46% of their nominal concentration. Retigabine is known to be oxygen and light sensitive, as well as acid and heat labile and not stable in methanolic solutions as it turns pink as an indication of degradation (260). Oxcarbazepine also was described as being a thermo-labile compound (261). Eslicarbazepine acetate was reported to degrade significantly in acid, base, neutral hydrolysis conditions as well (262). Lamotrigine and levetiracetam stability data indicates a slight increase in the concentration of some drugs over 24 hour period. This indicates practically no decomposition of AEDs (Table 3-6).

Table 3-6: Benchtop Stability of 17 AEDs for 24 Hours.

AEDs	Low *(n=3)*			High *(n=3)*		
	Measured Conc. (mg/L)		Recovery	Measured Conc. (mg/L)		Recovery
	T=0*	T=24	%	T=0*	T=24	%
CBZ	2.3	2.2	96	20.0	19.1	96
CBZO	17.0	16.4	96	28.0	28.1	100
ESL	**3.0**	**0.8**	**27**	**21.0**	**11.3**	**54**
GBP	2.3	2.3	100	20.0	18.5	93
LAC	13.0	13.3	102	23.0	23.9	104
LEV	115.0	132.2	115	185.0	203.0	110
LTG	1.7	2.0	117	13.0	15.2	117
OXC	**4.3**	**3.0**	**70**	**8.0**	**5.9**	**74**
PGR	2.0	1.9	95	16.0	15.5	97
PHT	2.1	2.1	100	21.0	20.9	100
p-HPPH	14.5	14.8	102	29.5	29.6	100
RTG	**2.0**	**1.8**	**78**	**9.5**	**7.3**	**76**
TIG	1.0	0.9	91	4.0	3.8	95
TPR	14.0	15.7	112	28.0	30.0	109
VIG	105.0	105.9	101	181.7	186.3	103
VPA	92.0	92.3	100	182.0	182.2	100
ZNS	9.0	9.5	106	17.0	17.5	102

*T refers to time in Hours.

In process stability: The extracted samples were stable in the autosampler (approximately 20ºC) for up to 24 hours. Although eslicarbazepine acetate, oxcarbazepine and retigabine are not stable in whole blood, their stability was acceptable after extraction and reconstitution in 13% methanol in water. Summary of the results are presented in Table 3-7.

Table 3-7: Autosampler Stability of 17 AEDs for 24 Hours.

AEDs	Low *(n=3)*			High *(n=3)*		
	Measured Conc. (mg/L)		Recovery	Measured Conc. (mg/L)		Recovery
	T=0*	T=24	%	T=0	T=24	%
CBZ	2.0	2.4	102	19.2	18.2	95
CBZO	15.7	16.0	102	27.4	28.7	105
ESL	2.9	3.1	108	20.9	20.7	99
GBP	2.5	2.6	104	21.9	21.8	99
LAC	13.9	13.8	99	23.2	23.6	101
LEV	112.9	115.6	102	170.0	187.0	110
LTG	1.7	1.6	94	12.8	14.0	109
OXC	4.3	4.1	96	8.0	8.1	101
PGR	1.6	1.9	116	15.8	16.4	104
PHT	2.5	2.5	100	19.9	20.5	103
p-HPPH	1.6	1.4	91	16.2	16.1	99
RTG	n/a	n/a	n/a	n/a	n/a	n/a
TIG	1.1	1.0	89	4.5	4.0	88
TPR	14.3	14.4	100	27.1	28.3	104
VIG	105.0	126.1	120	181.7	202.5	111
VPA	92.2	95.0	103	182.8	182.3	100
ZNS	9.1	9.2	101	16.4	17.3	105

* T refers to time in Hours.

Freeze-thaw cycles stability: All AEDs including oxcarbazepine and retigabine were stable after 3 freeze-thaw cycles at - 20ºC (Table 3-8). Eslicarbazepine acetate exhibited better stability in whole blood under freezing conditions which increased from 40 % for bench top stability to approximately 75 % in case of freeze-thaw stability.

Table 3-8: Freeze-Thaw Stability of 17 AEDs.

AEDs	Low *(n=3)* Measured Conc. (mg/L)				Recovery	High *(n=3)* Measured Conc. (mg/L)				Recovery
	T=0*	C1**	C2	C3	%	T=0	C1	C2	C3	%
CBZ	2.3	2.3	2.0	2.5	109	20.0	19.5	18.4	20.1	101
CBZO	17.0	16.2	16.2	17.8	105	28.0	27.1	27.7	27.8	99
ESL	**3.0**	**3.0**	**3.0**	**2.4**	**80**	**21.0**	**22.2**	**21.4**	**15.1**	**72**
GBP	2.3	2.3	2.6	2.3	100	23.0	19.1	21.0	23.2	101
LAC	13.0	13.6	13.8	15.1	116	23.0	24.6	24.1	23.6	103
LEV	115.0	131.0	112.1	139.3	121	185.0	204.7	190.7	199.8	108
LTG	1.7	2.1	1.8	1.6	94	13.0	16.1	13.6	16.2	125
OXC	4.3	4.4	4.1	4.7	108	8.0	8.8	8.2	7.7	96
PGR	1.9	2.0	1.8	1.7	89	16.0	15.8	15.9	17.1	107
PHT	2.1	2.2	2.5	1.9	90	21.0	20.6	20.5	21.5	102
p-HPPH	1.2	1.1	1.4	1.3	108	14.5	14.6	16.9	14.1	97
RTG	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
TIG	1.0	1.0	1.1	1.0	98	4.0	4.1	4.0	4.2	106
TPR	14.0	15.4	14.3	14.7	105	28.0	30.2	29.2	26.5	95
VIG	120.0	108.6	114.5	127.0	106	195.0	179.7	192.7	190.6	98
VPA	92.0	93.2	93.1	100.3	109	182.0	181.7	184.0	180.5	99
ZNS	9.0	9.4	8.8	10.6	118	17.0	18.2	17.9	17.5	103

* T=0: concentration measured at time zero. ** C refers to Freeze-thaw cycle number.

3.3.8 Dilution Integrity

The dilution integrity study showed the accuracy of two, four and ten times diluted samples to be within ± 15% of the nominal concentration and the precision was less than 11% (Table 3-9). These results conclude that the dilution of the concentrated whole blood sample up to ten times maintains integrity of AEDs.

Table 3-9: Dilution Integrity at 1:2, 1:5 and 1:10 Dilution Factors.

AEDs	Actual Conc. (mg/L)	QC4 (1in2 dilution, n=4)			QC4 (1in5 dilution, n=4)			QC4 (1in10 dilution, n=4)		
		Mean Measured Conc. (mg/L)	Accuracy (%)	Precision (%CV)	Mean Measured Conc. (mg/L)	Accuracy (%)	Precision (%CV)	Mean Measured Conc. (mg/L)	Accuracy (%)	Precision (%CV)
CBZ	100	82.1±3.4	82	4.1	98.1±2.4	98	2.4	117.6±0.8	115	0.7
CBZO	100	109.2±0.5	109	0.4	103.3±3.2	103	3.1	93.0±2.8	93	3.0
ESL	100	82.8±0.4	83	0.4	98.6±1.4	99	1.4	112.1±0.9	112	0.8
GBP	100	99.1±2.8	99	2.8	108±0.2	108	0.2	110.0±0.0	110	0.0
LAC	100	114.1±1.2	114	1.1	90.8±7.4	91	8.2	97.1±2.0	97	2.1
LEV	500	423.2±9.5	85	2.2	497.0±4.8	99	1.0	587.5±309	115	0.7
LTG	100	82.6±6.4	83	7.8	114.6±3.2	115	2.8	113.0±1.4	113	1.3
OXC	20	18.2±0.1	91	0.4	18.7±0.5	93	2.7	19.0±0.6	95	3.4
PGR	100	85±7.1	85	8.3	97.5±3.5	98	3.6	95.3±0.2	95	0.2
PHT	100	92.7±3.5	93	3.8	108.0±2.8	108	2.6	100.3±0.5	100	0.5
p-HPPH	100	88.2±0.1	88	0.1	88.6±0.4	89	0.4	95.3±1.2	95	1.2
RTG	20	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
TIG	20	19.4±0.3	97	1.7	22.8±0.3	114	1.2	19.2±2.1	96	11.0
TPR	100	91.9±1	92	1.1	97.7±2.1	98	2.1	112.5±3.5	113	3.1
VIG	500	434.3±0.5	88	0.1	510.0±21.2	102	4.2	575±35.4	35	6.1
VPA	500	488.3±7.1	98	1.5	551.9±19.9	110	3.6	558.3±58.9	112	10.6
ZNS	100	114±1.4	114	1.2	81.9±2.7	82	3.3	90.7±4.0	91	4.4

3.4 Conclusion

A simple, accurate and simultaneous analysis of 15 AEDs and 2 metabolites using LC/MS/MS triple quadruple was successfully developed and validated according to standard practices for method validation in forensic toxicology (SWGTOX) (242). The method is quantitative for 16 AEDs and qualitative for retigabine which did not pass the quantitative validation criteria for undetermined reasons.

The method developed for a small sample volume (100µL) used simple protein precipitation with methanol and achieved recoveries greater than 95% for all drugs (except retigabine, 33%) with acceptable matrix effect (within ± 25%). Good linearity, precision and accuracy were obtained for all drugs with $R^2 > 0.99$. Lamotrigine was successfully validated using quadratic curve.

In spite of the high cost of this method at the development stage, on a long-term basis the method would save on analyst time, cost of consumables and effort as it only requires a standard mix for the analysis of the 17 compounds. Rather than using many different methods to test for these substances individually, the same procedure can be used. It may also reveal the presence of drugs that have not been mentioned in the medical history either prescribed or misused.

Simultaneous methods can be a great advantage for routine laboratory work where consumables, time and effort saving are critical and a prerequisite to achieve targets and meet deadlines.

4 Short Term Stability Study of AEDs in Whole Blood

4.1 Introduction

Following method validation for the analysis of 15 most encountered antiepileptic drugs and two major metabolites (detailed in Chapter 3), the method was used to investigate the stability of these drugs (except retigabine) in whole blood under different storage conditions.

Stability studies are of great importance in forensic cases where it takes up to a few weeks between autopsy, sampling, drug screening and finally confirmation analysis. In some cases, quantitation analysis may not be requested until the case goes to court and is hence performed many weeks or months after the sample has been collected. Drug stability may lead to false negative results if the drug is not stable for example olanzapine where a loss of almost 100% at all storage temperatures was observed (263) or false positive results for example for GHB where higher concentration were reported in postmortem blood compared to freshly collected ones due to the in-vitro production of GHB during the storage time (264). Thus, knowledge of in-vitro drug stability in biological samples under different conditions is a prerequisite for the interpretation of the toxicological findings and to explain any discrepancies found between the initial quantitative results and those obtained in a second requested analysis (265).

A number of antiepileptic drug stability studies in serum/plasma, pharmaceutical bulk or blood collection devices have also been published (262, 266, 267). However, reports specifically addressing the stability of antiepileptic drugs in whole blood are relatively scarce compared with those for drugs of abuse, and if there are any published studies, they discuss the old generation of antiepileptic drugs such as carbamazepine, phenytoin and valproic acid (268, 269). Stability experiments for these drugs are often carried out as part of the bioanalytical method development and validation, and such data is very difficult to retrieve by a systematic literature search, unless the stability experiments are mentioned in the title or the abstract (270-273).

This would appear to be the first study to specifically address the stability for the new generation of antiepileptic drugs of interest in whole blood under different storage conditions.

4.2 Methodology

A short- term stability study was conducted using 100 mL drug free blood spiked with 16 AEDs at 3 different concentrations; 3, 20 and 40 mg/L for group 1; 1, 4 and 8 mg/L for group 2; and 20, 120 and 200 mg/L for group 3 (see 2.2.2.2 and 2.2.2.3 for QCs and solution preparation). Retigabine was excluded from this stability study due to its inconsistent response during method development and validation.

Freshly spiked QCs were initially extracted and analysed in triplicate and injected in duplicate using the validated method to establish time zero concentrations (detailed in 3.2.10). For each concentration, 100 µL aliquots were placed into labelled 1.5 mL Eppendorf tubes. The aliquots were stored under three different conditions; at room temperature (RT) about 18 ± 2 ºC, in the fridge at 4 ± 2 ºC, and in the freezer at -20 ± 2 ºC. In addition, an autosampler stability study was carried out over a 168 hour period.

Autosampler stability (~ 20 ºC) was evaluated by re-injecting extracted QCs at low and high concentrations; 3 and 40 mg/L for group 1; 1 and 8 mg/L for group 2; and 20 and 200 mg/L for group 3, after 24, 48, 72, 120 and 168 hours. Samples were stored on the autosampler over the study duration. In general, autosampler or in process stability was evaluated over the period of time required to analyse the samples on the instrument which is 20 minutes per sample in this case and can go up to 10 hours per batch. However, the stability study was extended up to 168 hours (7 days) in order to assess the stability of the processed samples. This is particularly significant when there is instrument down time and samples cannot be analysed directly after preparation or when there is a very long batch running. Assuming this study shows that the drugs are stable, then this would save the time needed to re-prepare the samples, save on consumables and solvents, and allow the same samples to be reinjected if needed with high accuracy.

Bench top stability at room temperature (18 ± 2) was investigated for 4 weeks. QCs were extracted in triplicate and injected in duplicate (n=6) on day 0, 1, 3, 7, 13, 23 and 30.

Fridge stability was evaluated for 10 weeks and freezer stability for 12 weeks. Samples were analysed in triplicate and injected in duplicate at 3 different concentrations on the first, third and seventh day during the first week, then once a week.

The concentrations of the analytes were calculated using freshly prepared calibration curves included in each batch. The values obtained were compared with the average time zero concentrations and the recovery of the remaining concentrations was calculated by dividing the final concentration obtained at the end of the stability study by the concentration at time zero and multiplying by 100.

4.3 Results and Discussion

As mentioned previously in 3.3.4, all drugs included in this study were proven to fulfil the accuracy acceptance criteria of being within 10 % of the target value during method validation with the exception of eslicarbazepine acetate and oxcarbazepine which exhibited a loss of about 40 % after 24 hours at room temperature. The stability results obtained in this study for AEDs in whole blood were comparable to their stability in serum/plasma and agreed with what has been published in the literature (262, 266-273).

4.3.1 Autosampler Stability

All AEDs exhibited good stability on the autosampler at 20ºC ± 2 with a recovery higher than 93% except tigabine and eslicarbazepine acetate which had recoveries of 85% and 87% respectively at the low concentration (Table 4-1). A slight increase in the concentration for some AEDs was observed such as carbamazepine epoxide (116% at low concentration and 124% at high concentration), carbamazepine (117% at high concentration), vigabatrin (123% at high concentration), lamotrigine (131% at low concentration and 111% at high concentration) and phenytoin (116% at low concentration).

Table 4-1: Stability Results of 16 AEDs in Whole Blood on The Autosampler Over 168 Hours at 2 Different Concentrations; 3 and 40 mg/L for Group 1; 4 and 8 mg/L for Group 2; and 20 and 200 mg/L for Group 3.

AED	QC1 (mg/L)						QC2 (mg/L)					
	T*=0	T=24	T=72	T=120	T=168	R%**	T=0	T=24	T=72	T=120	T=168	R%
CBZ	19.2	18.2	22.6	21.9	20.9	**109**	33.3	33.4	36.1	35.4	38.9	**117**
CBZO	15.7	16.0	20.1	19.3	18.2	**116**	27.4	28.7	30.6	29.9	33.9	**124**
ESL	20.9	20.7	24.2	23.2	18.1	**87**	37.0	37.9	37.3	37.4	37.4	**101**
GBP	22.0	21.8	22.1	22.0	21.4	**98**	41.0	41.0	43.0	40.6	40.7	**99**
LAC	13.9	13.8	17.4	15.3	14.1	**101**	23.2	23.6	22.9	25.5	24.7	**106**
LEV	112.9	115.6	139.7	125.8	115.0	**102**	170.1	186.6	208.2	196.2	185.5	**109**
LTG	12.8	14.0	17.3	13.6	16.7	**131**	19.1	21.0	18.2	20.6	21.2	**111**
OXC	4.3	4.1	5.3	4.2	4.0	**91**	8.0	8.1	8.0	8.6	7.9	**99**
PGR	15.8	16.4	15.9	17.5	17.0	**108**	30.1	30.9	31.8	32.2	32.3	**107**
PHT	20.0	20.5	26.9	21.2	23.3	**116**	40.4	40.5	42.9	44.3	42.0	**104**
HPPH	16.2	16.1	16.1	13.9	15.8	**97**	33.0	31.6	30.4	30.2	33.2	**101**
TIG	4.5	3.9	4.0	4.8	3.8	**85**	8.3	7.4	7.6	8.9	7.7	**93**
TPR	14.3	14.4	16.8	15.1	14.0	**98**	27.1	28.2	30.2	29.9	27.3	**101**
VIG	115.0	126.6	115.0	129.6	125.0	**109**	181.7	202.5	195.4	223.3	223.0	**123**
VPA	92.2	94.9	103.0	93.4	97.6	**106**	182.8	182.3	185.3	183.3	191.3	**105**
ZNS	9.1	9.2	9.1	9.2	8.9	**98**	16.4	17.3	18.0	16.2	16.9	**103**

* T represents time in hours.

** R%= Recovery or Remaining concentration after 168 hours.

4.3.2 Bench Top, Fridge and Freezer Stability

All AEDs exhibited good stability under various storage conditions during the investigated time frame. The recoveries of the remaining concentrations were within 20% accuracy of nominated concentrations for most drugs except eslicarbazepine acetate and oxcarbazepine.

Eslicarbazepine acetate instability at room temperature was reported earlier in the literature (207, 262). It is a prodrug which rapidly metabolises in the body to eslicarbazepine, R-licarbazepine and oxcarbazepine (Figure 4-1).

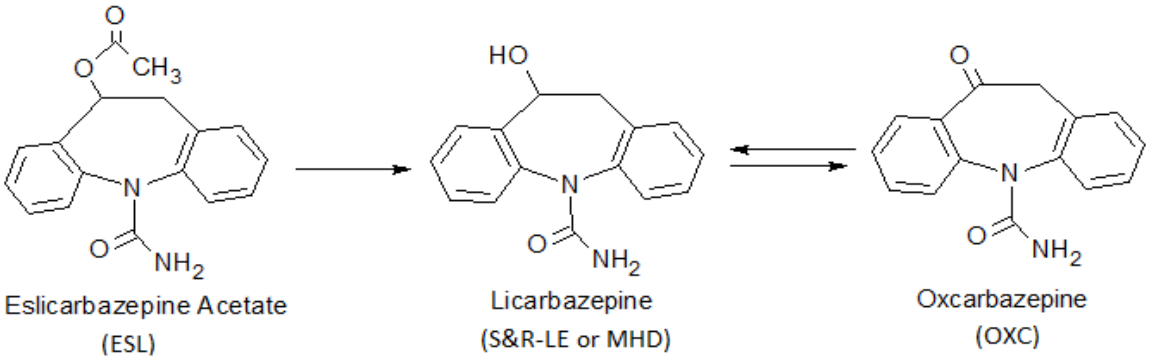


Figure 4-1: Eslicarbazepine Acetate and its Metabolites Structures.

Licarbazepine was tested for in this study as a racemic mix and the data is presented in Figure 4-2. By the end of the first week at room temperature eslicarbazepine acetate completely degraded (decreased from 8 mg/L to 0 mg/L) while the licarbazepine concentration increased from 0 to 5.6 mg/L at the end of the 4 week study duration. Although eslicarbazepine is metabolised to oxcarbazepine as well, no formation of oxcarbazepine was detected over the 4 week period.

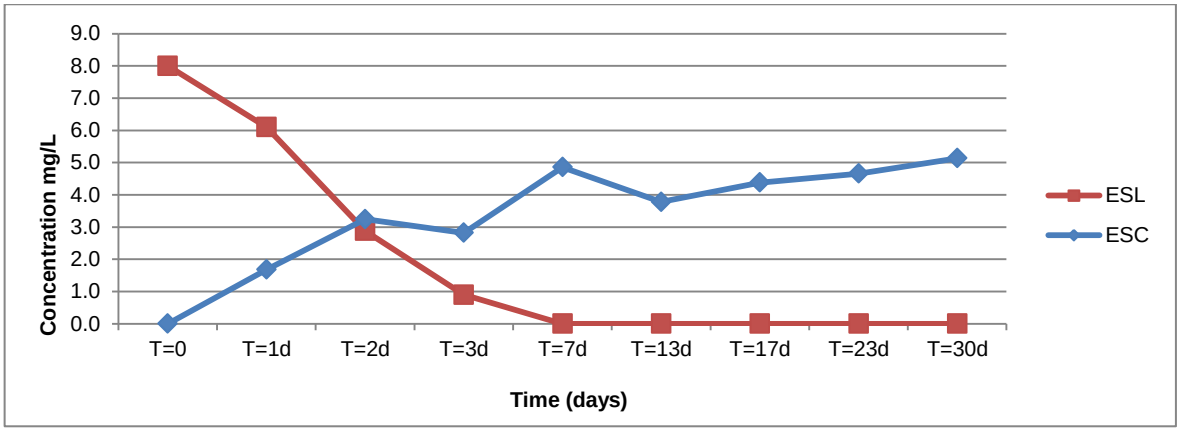


Figure 4-2: Eslicarbazepine Acetate and Licarbazepine Concentration Changes Over 4 Weeks at Room Temperature.

Similarly, oxcarbazepine results exhibited a quick degradation and the results were negative after 3 days storage at room temperature (Figure 4-3). Although, oxcarbazepine is metabolized to licarbazepine, this metabolite was not investigated in this study. By the end of the 10 week study duration, both eslicarbazepine acetate and oxcarbazepine exhibited a loss of more than 90 % of their nominated concentrations when stored at 4 ^{o}C. Nevertheless, they were stable at -20 °C (freezer stability) for 12 weeks.

Valproic acid, vigabatrin and zonisamide also lost about 30 - 40 % of their nominal concentrations after 4 week bench top stability at room temperature but they were stable in the fridge for 10 weeks and in freezer for 12 weeks.

Finally, results of calculated concentrations in each batch and the remaining concentrations presented as percentages at low, medium and high concentrations for bench top, fridge and freezer stabilities are detailed in Table 4-2 to Table 4-9.

4.1 Conclusion

This is the first stability study to specifically address the stability of 16 AEDs in whole blood. This study showed a good stability for antiepileptic drugs on the auto sampler for 168 hours, on the bench top at ~20 ºC for 4 weeks, in the fridge at ~4 ºC for 10 weeks and in the freezer at -20 ± 2 ºC for 12 weeks except for eslicarbazepine acetate and oxcarbazepine which both lost almost 100 % of their concentrations after 3 days bench top stability and 7 days fridge stability although they were stable in the freezer over the study duration.

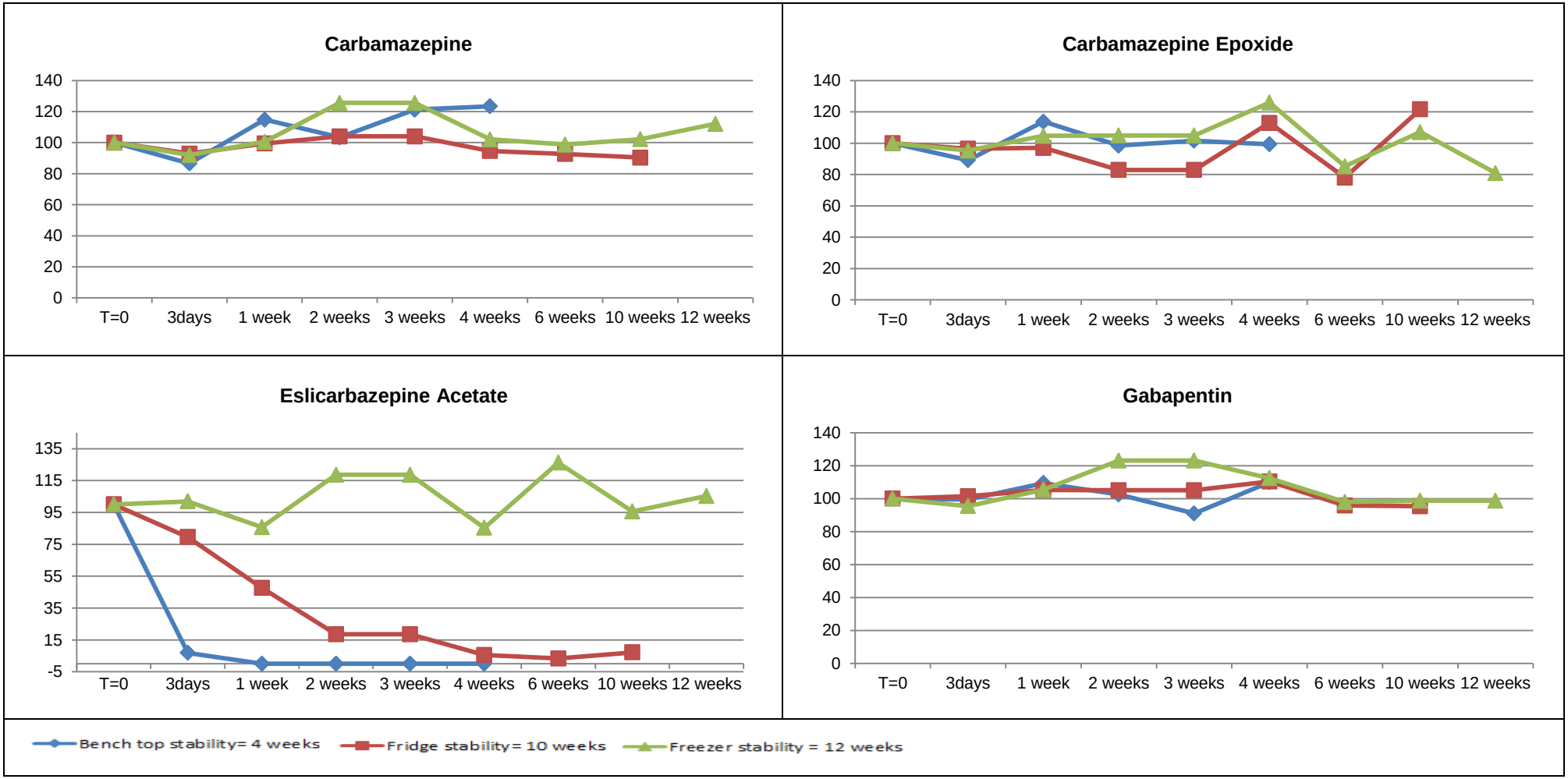


Figure 4-3: Bench Top, Fridge and Freezer Stability of AEDs in Whole Blood at 3 Different Concentrations; 3, 20 and 40 mg/L for Group 1; 1, 4 and 8 mg/L for Group 2; and 20, 120 and 200 mg/L for Group 3.

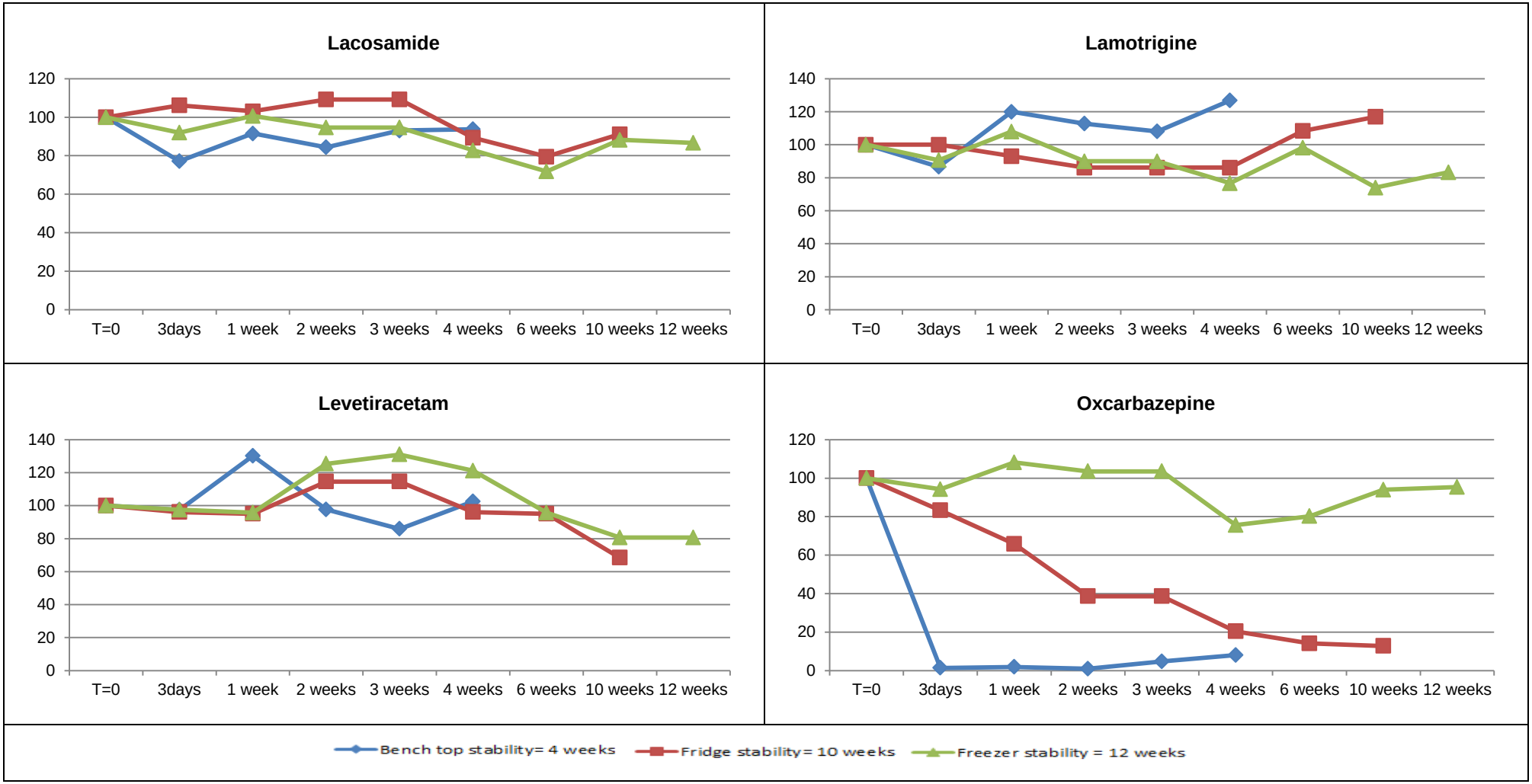


Figure 4-3: Bench Top, Fridge and Freezer Stability of AEDs in Whole Blood at 3 Different Concentrations; 3, 20 and 40 mg/L for Group 1; 1, 4 and 8 mg/L for Group 2; and 20, 120 and 200 mg/L for Group 3 (Continued…).

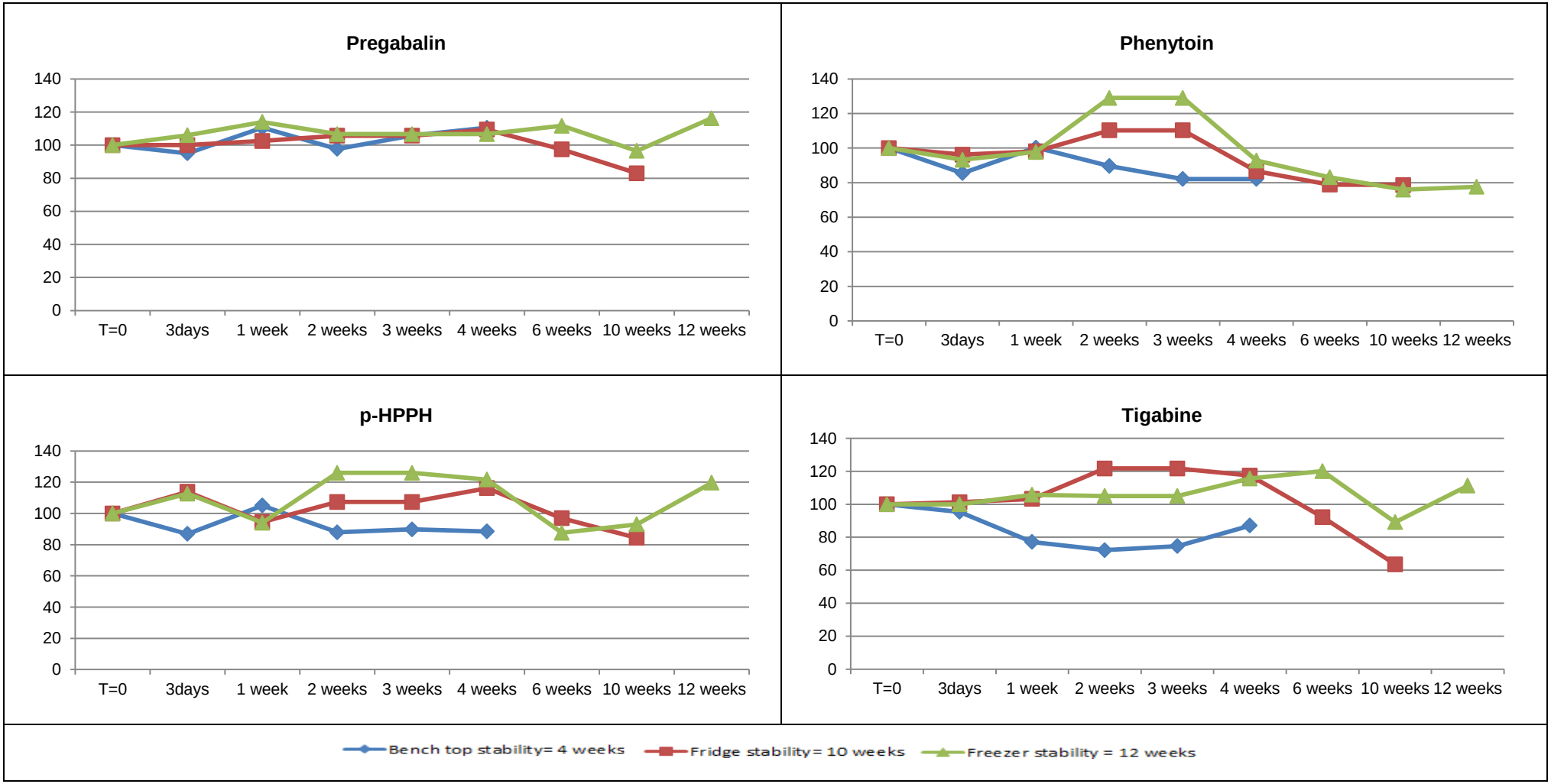


Figure 4-3: Bench Top, Fridge and Freezer Stability of AEDs in Whole Blood at 3 Different Concentrations; 3, 20 and 40 mg/L for Group 1; 1, 4 and 8 mg/L for Group 2; and 20, 120 and 200 mg/L for Group 3 (Continued…).

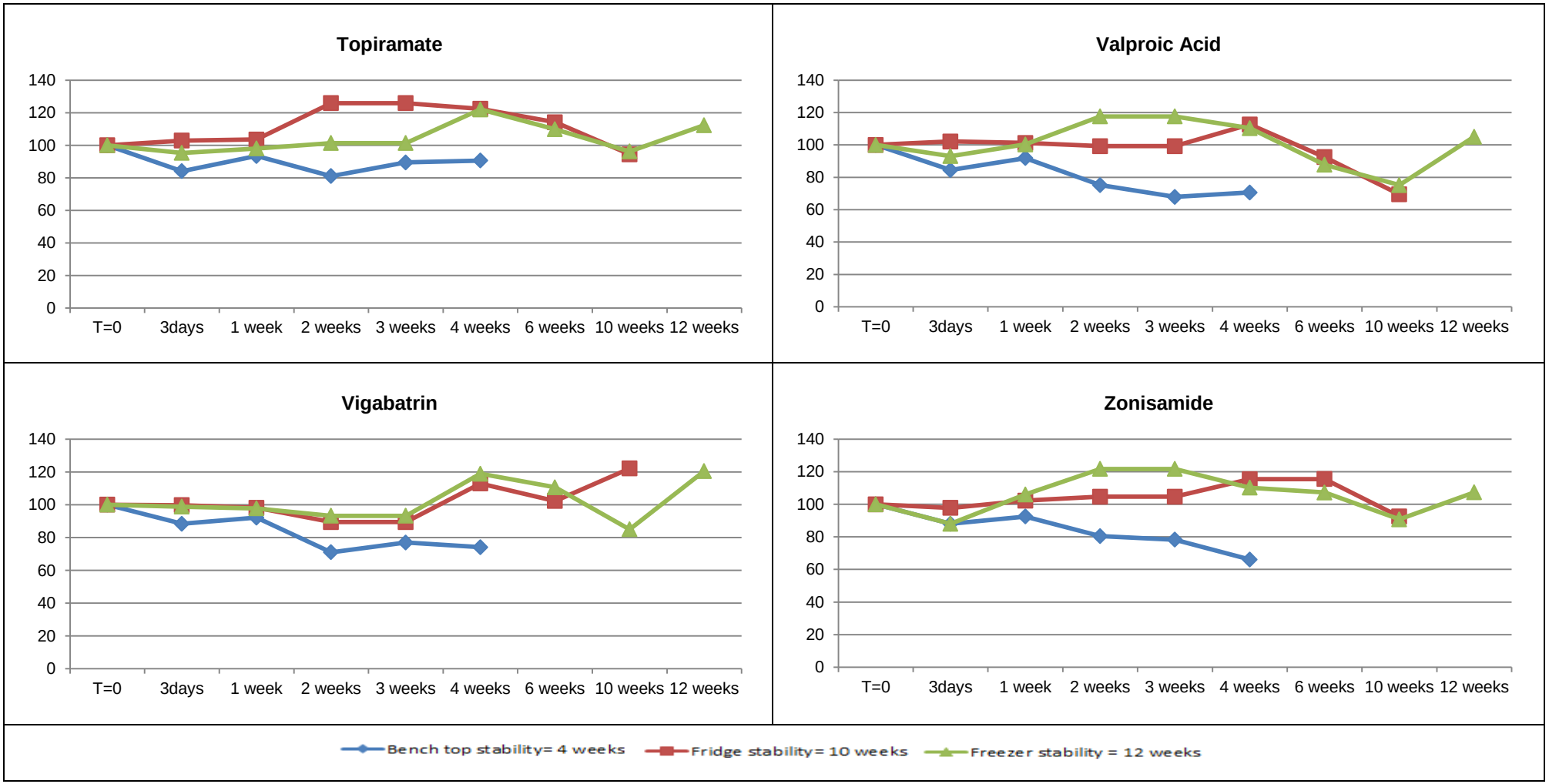


Figure 4-3: Bench Top, Fridge and Freezer Stability of AEDs in Whole Blood at 3 Different Concentrations; 3, 20 and 40 mg/L for Group 1; 1, 4 and 8 mg/L for Group 2; and 20, 120 and 200 mg/L for Group 3 (Continued…).

Table 4-2: Bench Top Stability Results of AEDs in Whole Blood at Low Concentrations (3 mg/L for Group 1; 1 mg/L for Group 2; and 20 mg/L for Group 3) Over 4 Week Period.

	AED	T=0	T=24	T=48	T=72	T=7d	T=13	T=17	T=23	T=30	R%
Mean QC1 (mg/L, n =6)	CBZ	10.0 ± 0.1	10.1 ± 0.3	11.9 ± 3.0	9.5 ± 0.8	11.1 ± 0.4	11.8 ± 1.1	13.8 ± 1.1	14.2 ± 0.6	12.0 ± 1.0	**120**
	CBZO	20.0 ± 0.3	20.2 ± 0.5	23.6 ± 4.5	19.5 ± 1.5	28.2 ± 0.6	22.2 ± 2.0	24.8 ± 1.7	24.5 ± 0.7	24.1 ± 1.3	**121**
	ESL	2.0 ± 0.0	1.5 ± 0.1	0.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	**0**
	GBP	5.0 ± 0.1	4.7 ± 0.2	5.8 ± 1.5	5.5 ± 1.4	4.4 ± 0.3	4.3 ± 1.1	4.6 ± 0.7	4.0 ± 0.3	5.3 ± 0.5	**106**
	LAC	15.0 ± 0.2	13.7 ± 0.4	16.0 ± 3.9	13.0 ± 1.2	19.4 ± 0.6	13.3 ± 1.9	14.0 ± 1.1	13.8 ± 0.4	16.2 ± 0.6	**108**
	LEV	50.0 ± 0.7	45.4 ± 1.1	55.0 ± 12.8	49.0 ± 8.7	68.1 ± 2.4	50.8 ± 7.5	51.0 ± 3.7	43.8 ± 1.9	50.3 ± 1.6	**101**
	LTG	10.0 ± 0.2	9.2 ± 0.3	10.8 ± 2.9	8.8 ± 1.1	13.0 ± 0.1	10.5 ± 1.8	11.6 ± 1.1	10.4 ± 0.2	11.6 ± 0.2	**116**
	OXC	2.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.1 ± 0.1	**7**
	PGR	5.0 ± 0.1	4.7 ± 0.2	5.6 ± 1.4	5.2 ± 1.2	4.3 ± 0.3	3.3 ± 0.9	4.1 ± 0.6	3.9 ± 0.3	3.6 ± 1.6	**72**
	PHT	12.0 ± 0.5	10.9 ± 0.4	12.8 ± 2.4	11.3 ± 1.9	14.0 ± 0.4	10.9 ± 0.9	12.6 ± 1.4	10.5 ± 0.6	11.2 ± 0.6	**93**
	HPPH	12.0 ± 0.4	11.7 ± 0.6	13.7 ± 2.4	12.0 ± 1.1	15.6 ± 0.5	12.2 ± 1.7	13.5 ± 0.9	11.8 ± 1.3	13.0 ± 0.9	**108**
	TIG	2.0 ± 0.0	2.0 ± 0.1	1.6 ± 0.3	1.7 ± 0.0	2.1 ± 0.0	3.0 ± 0.2	1.7 ± 0.3	2.0 ± 0.1	2.3 ± 0.1	**113**
	TPR	15.0 ± 0.3	13.4 ± 0.5	15.3 ± 2.9	12.4 ± 0.9	15.1 ± 0.6	11.6 ± 1.1	13.3 ± 1.1	13.7 ± 0.7	14.8 ± 0.8	**99**
	VIG	115.0 ± 1.0	79.9 ± 1.6	84.8 ± 16.4	74.8 ± 4.3	85.5 ± 0.8	73.5 ± 21.6	50.6 ± 0.5	82.1 ± 2.0	51.5 ± 11.8	**45**
	VPA	70.0 ± 0.0	58.4 ± 0.9	64.6 ± 10.7	55.7 ± 6.5	66.8 ± 1.3	46.6 ± 3.3	56.0 ± 4.4	48.1 ± 1.1	56.1 ± 4.1	**80**
	ZNS	7.0 ± 0.2	6.2 ± 0.4	6.6 ± 1.5	5.7 ± 0.8	6.7 ± 0.1	4.9 ± 0.6	6.0 ± 0.4	4.6 ± 0.2	5.5 ± 0.5	**78**

Table 4-3: Bench Top Stability Results of AEDs in Whole Blood at High Concentrations (40 mg/L for Group 1; 8 mg/L for Group 2; and 200 mg/L for Group 3) Over 4 Week Period

	AED	T=0	T=24	T=48	T=72	T=7d	T=13	T=17	T=23	T=30	R%
Mean QC2 (mg/L, n =6)	CBZ	40.0 ± 0.8	30.2 ± 0.7	35.7 ± 1.0	30.3 ± 1.0	45.9 ± 3.8	36.2 ± 1.4	42.1 ± 2.1	42.5 ± 1.5	47.3 ± 1.5	**118**
	CBZO	50.2 ± 0.9	45.6 ± 0.8	50.8 ± 0.8	44.8 ± 1.3	57.1 ± 2.3	49.4 ± 1.1	54.1 ± 2.5	51.0 ± 1.1	49.9 ± 3.3	**99**
	ESL	8.0 ± 0.3	6.1 ± 0.2	2.9 ± 0.1	0.9 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	**0**
	GBP	20.0 ± 0.2	19.8 ± 0.2	17.8 ± 0.4	19.2 ± 0.7	23.6 ± 1.0	21.4 ± 0.9	22.9 ± 0.7	19.9 ± 0.7	25.3 ± 0.6	**127**
	LAC	50.0 ± 3.4	40.6 ± 1.5	57.1 ± 1.6	40.7 ± 3.1	48.2 ± 3.7	44.5 ± 4.3	54.4 ± 2.5	49.0 ± 1.6	49.4 ± 0.8	**99**
	LEV	150.0 ± 2.3	136.3 ± 7.2	169.6 ± 4.2	146.1 ± 9.3	195.2 ± 13.3	146.4 ± 5.9	177.1 ± 10.1	128.8 ± 5.3	153.7 ± 5.4	**102**
	LTG	30.0 ± 1.6	26.6 ± 0.9	25.5 ± 1.0	25.5 ± 2.0	28.4 ± 2.6	26.7 ± 2.8	29.0 ± 2.4	25.6 ± 0.9	30.0 ± 2.2	**100**
	OXC	8.0 ± 0.7	0.6 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.2	0.7 ± 0.6	**8**
	PGR	20.0 ± 0.2	19.4 ± 0.5	16.7 ± 0.3	14.8 ± 0.6	20.8 ± 1.1	18.4 ± 0.9	20.8 ± 0.5	16.5 ± 0.5	19.7 ± 0.4	**98**
	PHT	40.0 ± 1.3	38.9 ± 1.0	39.9 ± 1.6	36.4 ± 1.4	42.7 ± 3.7	38.1 ± 0.3	41.9 ± 1.1	34.9 ± 0.8	34.9 ± 1.3	**87**
	HPPH	40.0 ± 1.0	40.1 ± 1.7	42.2 ± 0.6	37.0 ± 1.5	44.8 ± 2.5	37.4 ± 1.7	44.4 ± 0.9	38.3 ± 2.4	37.7 ± 0.7	**94**
	TIG	8.0 ± 0.1	7.5 ± 0.1	8.4 ± 0.2	8.0 ± 0.1	6.2 ± 0.5	5.3 ± 0.2	5.9 ± 0.4	6.0 ± 0.4	6.6 ± 0.2	**82**
	TPR	40.0 ± 1.3	43.8 ± 1.0	44.6 ± 1.0	40.3 ± 1.8	44.8 ± 2.0	38.9 ± 1.1	43.1 ± 1.3	42.9 ± 2.0	43.5 ± 0.4	**109**
	VIG	190.0 ± 5.0	194.0 ± 9.8	218.3 ± 3.6	188.6 ± 5.5	196.6 ± 1.8	151.6 ± 7.6	158.0 ± 0.6	164.3 ± 5.3	158.1 ± 11.5	**83**
	VPA	215.0 ± 3.8	197.0 ± 6.9	198.4 ± 3.4	180.5 ± 7.4	196.5 ± 2.4	160.8 ± 4.8	179.2 ± 5.1	145.1 ± 8.2	150.9 ± 5.1	**70**
	ZNS	20.0 ± 0.8	19.2 ± 1.2	20.0 ± 0.7	18.9 ± 1.6	19.8 ± 1.1	17.3 ± 0.9	19.4 ± 0.5	16.8 ± 0.6	14.2 ± 0.3	**71**

Table 4-4: Fridge Stability Results of AEDs in Whole Blood at Low Concentrations (3 mg/L for Group 1; 1 mg/L for Group 2; and 20 mg/L for Group 3) Over 10 Week Period.

	AED	Time=0	3 days	1 week	3 weeks	4 weeks	6 weeks	10 weeks	R %
Mean QC1 (mg/L, n =6)	CBZ	3.0 ± 0.1	2.0 ± 0.1	2.5 ± 0.2	3.2 ± 0.1	1.4 ± 0.1	2.3 ± 0.2	2.5 ± 0.0	**83**
	CBZO	2.0 ± 0.0	1.5 ± 0.1	1.7 ± 0.2	2.1 ± 0.0	2.5 ± 0.1	2.0 ± 0.2	2.3 ± 0.2	**114**
	ESL	3.0 ± 0.1	2.2 ± 0.0	1.3 ± 0.2	0.3 ± 0.1	0.5 ± 0.0	0.4 ± 0.2	0.0 ± 0.0	**0**
	GBP	2.0 ± 0.1	2.5 ± 0.0	2.3 ± 0.0	2.8 ± 0.1	2.7 ± 0.0	2.9 ± 0.0	2.3 ± 0.1	**115**
	LAC	2.0 ± 0.0	1.3 ± 0.1	1.1 ± 0.0	2.2 ± 0.0	1.2 ± 0.1	1.2 ± 0.0	2.5 ± 0.0	**125**
	LEV	20.0 ± 0.2	15.6 ± 0.2	15.3 ± 0.1	21.2 ± 0.2	15.6 ± 0.2	15.3 ± 0.1	12.7 ± 0.2	**64**
	LTG	2.0 ± 0.1	1.6 ± 0.3	1.5 ± 0.1	3.1 ± 0.1	3.0 ± 0.3	3.6 ± 0.1	2.3 ± 0.2	**116**
	OXC	2.0 ± 0.0	1.0 ± 0.0	0.7 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	**8**
	PGR	2.0 ± 0.0	1.7 ± 0.0	1.8 ± 0.0	2.3 ± 0.0	2.3 ± 0.0	2.3 ± 0.0	2.3 ± 0.0	**115**
	PHT	5.0 ± 0.2	4.5 ± 0.4	4.1 ± 0.1	4.2 ± 0.2	5.0 ± 0.4	4.3 ± 0.1	4.0 ± 0.7	**80**
	HPPH	2.0 ± 0.1	1.3 ± 0.1	1.4 ± 0.0	2.6 ± 0.1	3.0 ± 0.1	1.7 ± 0.0	2.3 ± 0.2	**115**
	TIG	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.2	1.5 ± 0.1	1.3 ± 0.0	1.2 ± 0.2	0.9 ± 0.0	**90**
	TPR	3.0 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.1 ± 0.1	2.7 ± 0.1	**89**
	VIG	n/a	n/a	n/a	n/a	n/a	n/a	n/a	**n/a**
	VPA	25.0 ± 0.2	17.8 ± 0.4	16.8 ± 0.2	13.9 ± 0.2	18.8 ± 0.4	16.6 ± 0.2	21.0 ± 0.2	**84**
	ZNS	2.0 ± 0.1	1.4 ± 0.2	1.9 ± 0.3	1.8 ± 0.1	2.4 ± 0.2	2.7 ± 0.3	2.3 ± 0.1	**117**

Table 4-5: Fridge Stability Results of AEDs in Whole Blood at Medium Concentrations (20 mg/L for Group 1; 4 mg/L for Group 2; and 120 mg/L for Group 3) Over 10 Week Period.

	AED	Time=0	3 days	1 week	3 weeks	4 weeks	6 weeks	10 weeks	R %
Mean QC2 (mg/L, n =6)	CBZ	20.0 ± 0.4	18.6 ± 0.7	19.9 ± 0.8	20.8 ± 0.4	18.9 ± 0.7	18.5 ± 0.8	18.1 ± 0.1	**90**
	CBZO	20.0 ± 0.4	16.4 ± 0.6	16.5 ± 0.6	14.1 ± 0.4	19.2 ± 0.6	13.3 ± 0.6	20.7 ± 0.4	**103**
	ESL	20.0 ± 0.2	16.7 ± 0.1	10.0 ± 0.2	3.9 ± 0.2	1.2 ± 0.1	0.7 ± 0.2	1.5 ± 0.0	**8**
	GBP	20.0 ± 0.2	21.3 ± 0.6	22.1 ± 0.2	22.1 ± 0.2	23.2 ± 0.6	20.1 ± 0.2	20.0 ± 0.4	**100**
	LAC	15.0 ± 0.6	13.8 ± 0.2	13.4 ± 0.5	15.2 ± 0.6	11.6 ± 0.2	10.3 ± 0.5	11.8 ± 0.3	**79**
	LEV	115.0 ± 4.4	110.5 ± 0.3	109.3 ± 0.8	131.8 ± 4.4	110.5 ± 0.3	109.3 ± 0.8	78.7 ± 1.7	**68**
	LTG	15.0 ± 0.8	13.0 ± 0.2	12.1 ± 0.6	22.4 ± 0.8	22.2 ± 0.2	14.1 ± 0.6	19.4 ± 0.9	**129**
	OXC	4.0 ± 0.1	3.6 ± 0.1	2.8 ± 0.1	1.7 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	**14**
	PGR	15.0 ± 0.2	16.0 ± 0.4	16.4 ± 0.2	16.9 ± 0.2	17.5 ± 0.4	15.6 ± 0.2	14.3 ± 0.3	**95**
	PHT	20.0 ± 1.7	20.2 ± 0.8	20.6 ± 1.6	23.2 ± 1.7	18.2 ± 0.8	16.6 ± 1.6	16.5 ± 1.2	**83**
	HPPH	15.0 ± 0.5	16.5 ± 0.3	13.7 ± 0.9	15.6 ± 0.5	16.8 ± 0.3	14.1 ± 0.9	12.2 ± 0.3	**82**
	TIG	4.0 ± 0.2	4.1 ± 0.1	4.1 ± 0.5	4.9 ± 0.2	4.7 ± 0.1	3.7 ± 0.5	2.5 ± 0.1	**64**
	TPR	15.0 ± 0.6	14.4 ± 0.4	14.5 ± 0.8	17.6 ± 0.6	17.1 ± 0.4	16.0 ± 0.8	13.2 ± 0.4	**88**
	VIG	115.0 ± 1.6	118.6 ± 3.8	115.6 ± 3.0	96.4 ± 1.6	136.1 ± 3.8	115.8 ± 3.0	129.0 ± 5.9	**112**
	VPA	115.0 ± 2.6	94.0 ± 1.0	93.1 ± 3.7	91.3 ± 2.6	103.7 ± 1.0	85.1 ± 3.7	63.9 ± 1.2	**56**
	ZNS	8.0 ± 0.2	8.8 ± 0.2	9.2 ± 0.4	9.4 ± 0.2	10.4 ± 0.2	10.4 ± 0.4	8.3 ± 0.4	**104**

Table 4-6: Fridge Stability Results of AEDs in Whole Blood at High Concentrations (40 mg/L for Group 1; 8 mg/L for Group 2; and 200 mg/L for Group 3) Over 10 Week Period.

	AED	Time=0	3 days	1 week	3 weeks	4 weeks	6 weeks	10 weeks	R %
Mean QC3 (mg/L, n =6)	CBZ	30.0 ± 0.8	31.6 ± 1.3	33.1 ± 0.4	35.7 ± 0.8	31.7 ± 1.3	31.8 ± 0.4	30.0 ± 0.4	**100**
	CBZO	30.0 ± 0.7	25.0 ± 1.1	28.4 ± 0.5	24.5 ± 0.7	29.3 ± 1.1	23.4 ± 0.5	33.5 ± 0.8	**112**
	ESL	30.0 ± 0.3	31.3 ± 0.2	20.4 ± 0.3	10.1 ± 0.3	4.0 ± 0.2	2.6 ± 0.3	2.3 ± 0.0	**8**
	GBP	40.0 ± 0.4	39.6 ± 0.6	41.5 ± 0.3	39.5 ± 0.4	41.8 ± 0.6	36.7 ± 0.3	35.3 ± 0.4	**88**
	LAC	20.0 ± 0.2	22.8 ± 0.4	22.5 ± 0.2	28.6 ± 0.2	20.1 ± 0.4	17.8 ± 0.2	18.3 ± 0.4	**91**
	LEV	190.0 ± 3.6	179.4 ± 0.8	176.7 ± 0.5	215.8 ± 3.6	179.4 ± 0.8	176.7 ± 0.5	135.9 ± 2.8	**72**
	LTG	30.0 ± 0.6	20.1 ± 1.2	19.0 ± 0.6	34.3 ± 0.6	31.6 ± 1.2	20.3 ± 0.6	28.1 ± 1.5	**94**
	OXC	8.0 ± 0.2	6.8 ± 0.1	5.2 ± 0.1	3.6 ± 0.2	1.8 ± 0.1	0.9 ± 0.1	0.4 ± 0.0	**5**
	PGR	30.0 ± 0.3	30.4 ± 0.5	30.8 ± 0.2	30.2 ± 0.3	31.1 ± 0.5	28.1 ± 0.2	27.5 ± 0.2	**92**
	PHT	40.0 ± 2.0	40.1 ± 1.7	40.2 ± 0.6	44.7 ± 2.0	30.0 ± 1.7	28.2 ± 0.6	26.4 ± 1.0	**66**
	HPPH	30.0 ± 1.0	32.2 ± 0.6	27.6 ± 0.4	30.9 ± 1.0	29.4 ± 0.6	27.8 ± 0.4	21.6 ± 0.6	**72**
	TIG	8.0 ± 0.3	7.7 ± 0.1	7.9 ± 0.1	9.0 ± 0.3	8.0 ± 0.1	8.1 ± 0.1	3.9 ± 0.2	**49**
	TPR	30.0 ± 0.5	28.3 ± 0.5	27.5 ± 0.2	33.9 ± 0.5	31.9 ± 0.5	30.0 ± 0.2	23.7 ± 0.4	**79**
	VIG	190.0 ± 3.7	194.5 ± 9.7	191.3 ± 4.0	174.4 ± 3.7	220.3 ± 9.7	199.4 ± 4.0	223.2 ± 3.0	**117**
	VPA	190.0 ± 1.6	182.3 ± 2.1	180.8 ± 2.6	174.2 ± 1.6	189.0 ± 2.1	157.1 ± 2.6	109.3 ± 2.0	**58**
	ZNS	15.0 ± 0.2	17.2 ± 0.2	16.8 ± 0.3	17.9 ± 0.2	18.5 ± 0.2	18.2 ± 0.3	13.9 ± 0.2	**93**

Table 4-7: Freezer Stability Results of AEDs in Whole Blood at Low Concentrations (3 mg/L for Group 1; 1 mg/L for Group 2; and 20 mg/L for Group 3) Over 12 Week Period.

	AED	Time=0	1day	3days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	10 weeks	12 weeks	R%
Mean QC1 (mg/L, n =6)	CBZ	3.0 ± 0.0	2.3 ± 0.2	2.0 ± 0.1	2.5 ± 0.1	2.3 ± 0.0	3.4 ± 0.2	1.7 ± 0.1	2.5 ± 0.1	4.6 ± 0.0	3.3 ± 0.1	**110**
	CBZO	2.0 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	1.7 ± 0.1	2.2 ± 0.1	2.8 ± 0.1	2.1 ± 0.1	3.3 ± 0.1	1.5 ± 0.1	**74**
	ESL	4.0 ± 0.1	3.0 ± 0.2	3.0 ± 0.3	2.4 ± 0.1	2.9 ± 0.1	3.6 ± 0.2	2.9 ± 0.3	5.1 ± 0.1	4.1 ± 0.1	3.9 ± 0.2	**98**
	GBP	3.0 ± 0.1	2.3 ± 0.2	2.6 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	3.0 ± 0.2	2.8 ± 0.1	2.9 ± 0.1	4.2 ± 0.1	2.9 ± 0.1	**98**
	LAC	2.0 ± 0.0	1.3 ± 0.2	1.4 ± 0.0	1.1 ± 0.0	1.2 ± 0.0	2.4 ± 0.2	1.2 ± 0.0	1.4 ± 0.0	3.0 ± 0.0	2.0 ± 0.1	**101**
	LEV	20.0 ± 0.3	23.7 ± 1.8	17.4 ± 0.1	17.6 ± 0.1	15.2 ± 0.3	25.4 ± 1.8	17.6 ± 0.1	15.2 ± 0.1	17.6 ± 0.3	17.6 ± 0.1	**88**
	LTG	2.0 ± 0.1	2.1 ± 0.3	1.8 ± 0.3	1.6 ± 0.2	1.8 ± 0.1	1.8 ± 1.8	3.5 ± 0.3	1.8 ± 0.2	1.4 ± 0.1	2.1 ± 0.2	**105**
	OXC	2.0 ± 0.1	1.9 ± 0.1	2.4 ± 0.1	2.2 ± 0.0	2.2 ± 0.1	2.0 ± 0.1	1.9 ± 0.1	2.0 ± 0.0	2.1 ± 0.1	2.3 ± 0.1	**115**
	PGR	2.0 ± 0.0	2.0 ± 0.1	1.8 ± 0.1	1.7 ± 0.0	1.8 ± 0.0	2.5 ± 0.1	2.4 ± 0.1	2.4 ± 0.0	3.9 ± 0.0	2.3 ± 0.0	**115**
	PHT	4.0 ± 1.4	4.2 ± 0.3	4.5 ± 0.2	3.9 ± 1.3	4.0 ± 1.4	4.4 ± 0.3	4.9 ± 0.2	4.9 ± 1.3	5.6 ± 1.4	4.9 ± 1.0	**122**
	HPPH	3.0 ± 0.1	3.2 ± 0.2	3.4 ± 0.2	3.2 ± 0.3	2.5 ± 0.1	2.8 ± 0.2	3.3 ± 0.2	1.8 ± 0.3	3.2 ± 0.1	3.6 ± 0.3	**120**
	TIG	1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.0	0.9 ± 0.0	1.5 ± 0.1	1.1 ± 0.1	1.4 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	**116**
	TPR	2.0 ± 0.0	1.3 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.6 ± 0.0	2.7 ± 0.1	2.4 ± 0.1	2.2 ± 0.1	3.9 ± 0.0	2.4 ± 0.0	**118**
	VIG	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	**n/a**
	VPA	20.0 ± 0.2	17.4 ± 0.8	17.8 ± 0.4	16.7 ± 0.2	16.1 ± 0.2	15.0 ± 0.8	18.4 ± 0.4	17.3 ± 0.2	21.0 ± 0.2	17.9 ± 0.5	**90**
	ZNS	2.0 ± 0.0	1.2 ± 0.1	1.4 ± 0.1	1.1 ± 0.3	1.5 ± 0.0	1.8 ± 0.1	2.3 ± 0.1	2.9 ± 0.3	2.7 ± 0.0	2.7 ± 0.1	**137**

Table 4-8: Freezer Stability Results of AEDs in Whole Blood at Medium Concentrations (20 mg/L for Group 1; 4 mg/L for Group 2; and 120 mg/L for Group 3) Over 12 Week Period.

AED		Time=0	1day	3days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	10 weeks	12 weeks	R%
Mean QC2 (mg/L, n =6)	CBZ	20.0 ± 0.3	19.5 ± 3.0	18.4 ± 0.7	20.1 ± 0.5	20.3 ± 0.3	25.1 ± 3.0	20.4 ± 0.7	19.8 ± 0.5	20.4 ± 0.3	22.4 ± 0.8	**112**
	CBZO	15.0 ± 0.4	16.2 ± 2.3	16.2 ± 0.6	17.8 ± 0.4	18.1 ± 0.4	17.8 ± 2.3	21.4 ± 0.6	14.5 ± 0.4	20.9 ± 0.4	13.8 ± 0.5	**92**
	ESL	20.0 ± 0.3	22.2 ± 3.4	21.4 ± 0.9	15.1 ± 0.9	21.9 ± 0.3	26.9 ± 3.4	17.9 ± 0.9	28.2 ± 0.9	20.1 ± 0.3	22.1 ± 0.7	**111**
	GBP	20.0 ± 0.4	19.1 ± 3.9	21.0 ± 0.3	23.2 ± 0.4	21.0 ± 0.4	27.1 ± 3.9	24.7 ± 0.3	21.5 ± 0.4	21.7 ± 0.4	21.7 ± 0.3	**109**
	LAC	20.0 ± 0.3	13.6 ± 2.5	13.8 ± 0.3	15.1 ± 0.4	13.7 ± 0.3	20.9 ± 2.5	12.4 ± 0.3	10.8 ± 0.4	13.2 ± 0.3	21.2 ± 0.3	**106**
	LEV	115.0 ± 1.7	131.0 ± 17.6	112.1 ± 0.7	139.3 ± 0.6	110.1 ± 1.7	163.0 ± 17.6	139.3 ± 0.7	110.1 ± 0.6	92.8 ± 1.7	92.8 ± 0.4	**81**
	LTG	15.0 ± 1.0	16.1 ± 2.3	13.6 ± 0.6	16.2 ± 0.6	14.4 ± 1.0	13.2 ± 2.3	12.5 ± 0.6	14.7 ± 0.6	12.2 ± 1.0	13.8 ± 0.9	**92**
	OXC	4.0 ± 0.1	4.4 ± 1.4	4.1 ± 0.2	4.7 ± 0.2	4.2 ± 0.1	4.5 ± 1.4	3.5 ± 0.2	3.9 ± 0.2	4.0 ± 0.1	4.5 ± 0.4	**113**
	PGR	15.0 ± 0.1	15.8 ± 2.9	15.9 ± 0.3	17.1 ± 0.4	16.6 ± 0.1	21.0 ± 2.9	18.8 ± 0.3	16.8 ± 0.4	14.5 ± 0.1	17.4 ± 0.1	**116**
	PHT	20.0 ± 0.4	20.6 ± 3.7	20.5 ± 2.1	21.5 ± 2.2	20.1 ± 0.4	28.4 ± 3.7	20.4 ± 2.1	18.3 ± 2.2	16.7 ± 0.4	17.1 ± 1.3	**85**
	HPPH	14.5 ± 0.5	14.6 ± 3.1	16.9 ± 0.8	14.1 ± 5.8	13.3 ± 0.5	19.9 ± 3.1	18.2 ± 0.8	13.1 ± 5.8	13.9 ± 0.5	17.9 ± 1.0	**124**
	TIG	4.0 ± 0.2	4.1 ± 0.9	4.0 ± 0.2	4.2 ± 0.1	4.0 ± 0.2	6.3 ± 0.9	4.6 ± 0.2	4.8 ± 0.1	3.6 ± 0.2	4.5 ± 0.1	**111**
	TPR	15.0 ± 0.3	15.4 ± 3.3	14.3 ± 0.4	14.7 ± 0.4	13.6 ± 0.3	22.3 ± 3.3	18.3 ± 0.4	16.5 ± 0.4	14.4 ± 0.3	16.8 ± 0.3	**112**
	VIG	120.0 ± 4.0	108.6 ± 13.8	114.5 ± 5.2	127.0 ± 3.9	131.6 ± 4.0	120.4 ± 13.8	144.7 ± 5.2	123.7 ± 3.9	156.8 ± 4.0	139.6 ± 1.3	**116**
	VPA	115.0 ± 1.0	93.2 ± 17.6	93.1 ± 2.7	100.3 ± 1.6	92.3 ± 1.0	117.6 ± 17.6	110.4 ± 2.7	87.7 ± 1.6	114.2 ± 1.0	131.5 ± 1.1	**114**
	ZNS	10.0 ± 0.2	9.4 ± 1.8	8.8 ± 0.2	10.6 ± 0.3	8.5 ± 0.2	12.2 ± 1.8	11.0 ± 0.2	10.7 ± 0.3	9.1 ± 0.2	10.7 ± 0.2	**107**

Table 4-9: Freezer Stability Results of AEDs in Whole Blood at High Concentrations (40 mg/L for Group 1; 8 mg/L for Group 2; and 200 mg/L for Group 3) Over 12 Week Period.

	AED	Time=0	1day	3days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	10 weeks	12 weeks	R%
Mean QC3 (mg/L, n =6)	CBZ	40.0 ± 0.7	34.8 ± 3.1	31.0 ± 1.1	34.0 ± 0.9	34.4 ± 0.7	34.5 ± 3.1	34.6 ± 1.1	32.9 ± 0.9	33.6 ± 0.7	36.6 ± 0.4	**92**
	CBZO	30.0 ± 0.9	27.1 ± 2.3	27.7 ± 1.0	27.8 ± 0.8	29.4 ± 0.9	25.0 ± 2.3	33.9 ± 1.0	25.2 ± 0.8	33.8 ± 0.9	24.0 ± 0.4	**80**
	ESL	40.0 ± 1.2	37.5 ± 3.1	38.2 ± 0.9	23.6 ± 1.4	37.2 ± 1.2	37.3 ± 3.1	29.0 ± 0.9	50.2 ± 1.4	32.9 ± 1.2	35.8 ± 0.9	**90**
	GBP	40.0 ± 1.3	34.1 ± 3.8	40.6 ± 0.4	38.3 ± 0.7	39.1 ± 1.3	39.8 ± 3.8	44.1 ± 0.4	39.4 ± 0.7	37.8 ± 1.3	39.1 ± 0.5	**98**
	LAC	30.0 ± 0.4	24.6 ± 2.5	24.1 ± 0.4	23.6 ± 0.4	23.7 ± 0.4	28.6 ± 2.5	21.0 ± 0.4	18.4 ± 0.4	19.6 ± 0.4	32.7 ± 0.7	**109**
	LEV	190.0 ± 3.0	204.7 ± 15.8	190.7 ± 1.1	199.8 ± 0.7	185.8 ± 3.0	217.4 ± 15.8	199.8 ± 1.1	185.8 ± 0.7	152.6 ± 3.0	152.6 ± 1.4	**80**
	LTG	30.0 ± 1.4	27.5 ± 2.0	20.7 ± 0.8	24.5 ± 0.7	22.8 ± 1.4	33.8 ± 2.0	31.3 ± 0.8	22.5 ± 0.7	30.4 ± 1.4	35.3 ± 2.4	**118**
	OXC	8.0 ± 0.7	8.8 ± 1.1	8.2 ± 0.3	7.7 ± 0.3	8.4 ± 0.7	8.0 ± 1.1	8.5 ± 0.3	6.7 ± 0.3	7.5 ± 0.7	8.2 ± 0.3	**103**
	PGR	30.0 ± 0.7	30.1 ± 3.0	31.2 ± 0.4	28.0 ± 0.7	31.0 ± 0.7	30.7 ± 3.0	33.0 ± 0.4	30.4 ± 0.7	24.4 ± 0.7	31.7 ± 0.4	**106**
	PHT	40.0 ± 1.1	40.6 ± 3.6	41.1 ± 0.7	37.6 ± 0.7	40.3 ± 1.1	41.0 ± 3.6	31.5 ± 0.7	28.7 ± 0.7	28.5 ± 1.1	28.2 ± 0.4	**70**
	HPPH	29.5 ± 1.0	30.0 ± 2.8	34.6 ± 0.6	24.7 ± 0.5	29.4 ± 1.0	29.6 ± 2.8	31.5 ± 0.6	28.5 ± 0.5	23.7 ± 1.0	31.7 ± 0.2	**107**
	TIG	8.0 ± 0.1	7.3 ± 0.8	8.0 ± 0.1	7.5 ± 0.2	8.0 ± 0.1	9.1 ± 0.8	8.3 ± 0.1	8.1 ± 0.2	5.5 ± 0.1	7.8 ± 0.2	**98**
	TPR	30.0 ± 0.9	30.2 ± 2.8	29.2 ± 0.5	26.5 ± 0.3	27.3 ± 0.9	33.2 ± 2.8	33.1 ± 0.5	30.7 ± 0.3	24.5 ± 0.9	31.5 ± 0.8	**105**
	VIG	190.0 ± 5.9	179.7 ± 24.3	192.7 ± 9.2	190.6 ± 10.3	207.6 ± 5.9	181.9 ± 24.3	231.8 ± 9.2	215.8 ± 10.3	215.7 ± 5.9	215.7 ± 17.4	**114**
	VPA	190.0 ± 3.3	181.7 ± 15.2	184.0 ± 0.8	180.5 ± 1.5	182.7 ± 3.3	175.3 ± 15.2	201.4 ± 0.8	162.9 ± 1.5	113.6 ± 3.3	242.2 ± 5.2	**127**
	ZNS	20.0 ± 0.6	18.2 ± 1.7	17.9 ± 0.3	17.5 ± 0.4	16.1 ± 0.6	17.6 ± 1.7	19.3 ± 0.3	18.3 ± 0.4	15.1 ± 0.6	18.6 ± 0.7	**93**

5 Determination of 22 AEDs in Whole Blood, Serum and Plasma Using LC/MS/MS: Analytical Method Transfer Challenges and Considerations.

5.1 Introduction

Method transfer can occur within the same organization or between analytical laboratories and the transferred method validation can range from a check for accuracy and precision to a full validation depending on the analysis requirements and the purpose of the transferred method (250). Unfortunately, very limited information about method transfer protocols or challenges is available in the literature (274).

The aim of this project was to transfer the method developed at Forensic Medicine and Science lab (FMS), University of Glasgow, (Glasgow, UK) to NMS lab (PA, USA) and investigate the main factors required to be taken into consideration when transferring the LC/MS/MS method from one instrument to another or from one lab to another. As part of any method transfer, it is advised to test the same quality controls to compare the original and transferred methods. However, this was not applicable in this study due to the short time of the project which was not sufficient to arrange for QCs to be shipped from FMS lab to the NMS lab. Authentic sample transfer from NMS lab to FMS was also not possible due to ethical approval required by University of Glasgow and could not be obtained on time.

However, in order to verify the transferred method, 467 samples that had previously been tested at NMS lab, were reanalyzed using the method. The obtained results were compared with NMS lab values and statistically evaluated using Pearson correlation and Band-Altman plots. The data is discussed in details in Chapter 6.

The method was transferred and a re-validation was conducted for 22 AEDs. These included the 17 AEDs tested in the first developed method as well as a further 5 AEDs added as requested by NMS lab due to their high availability in the US market. These 5 drugs were phenobarbital, S-licarbazepine (eslicarbazepine acetate and oxcarbazepine metabolite), N-acetyl retigabine (retigabine metabolite), rufinamide and stiripentol.

Fosphenytoin was also one of the requested drugs but this is a prodrug of phenytoin which was already included in the method.

Due to the high number of therapeutic drug monitoring (TDM) analysis carried out by NMS lab using serum and plasma, it was decided to extend the method to include serum and plasma which made the method suitable for use in TDM as well as postmortem blood in forensic cases.

5.2 Materials and Methods

5.2.1 Materials

Eslicarbazepine acetate (ESL) was purchased from Santa Cruz Biotechnology (Texas, USA). S-Licarbazepine (S-LC), retigabine (RTG), N-acetyl retigabine (NA-RTG), phenobarbital (PBT), rufinamide (RFM), and gabapentin-D_{10} (GBP-D_{10}) were purchased from Cerilliant (Texas, USA). Lacosamide (LAC), pregabalin (PGR) and tigabine (TIG) were obtained from LGC standards (Canada, USA). Gabapentin (GBP), vigabatrin (VIG), valproic acid (VPA), levetiracetam (LEV), carbamazepine (CBZ), carbamazepine 10,11-epoxide (CBZO), oxcarbazepine (OXC), zonisamide (ZNS), topiramate(TPR), phenytoin (PHT), 5-(3-Hydroxyphenyl)-5-phenylhydantoin (p-HPPH), stiripentol (STP), lamotrigine (LTG), tolbutamide (TUB), 10- 11 dihydrocarbamazepine (CBZ-DiOH) and ammonium acetate (HPLC grade) were purchased from Sigma Aldrich (Missouri, USA). Methanol (HPLC grade) was supplied by VWR International Ltd (Philadelphia, USA). Double distilled water was obtained from the in-house Millipore® system.

5.2.2 Blank Blood and Biological Samples

Human blank whole blood, plasma and serum were obtained from Golden West Biological Inc® (California, USA). Postmortem blood (n=7), plasma (n=7) and serum samples (n=453) were kindly provided by NMS lab (PA, USA) collected over a two month period and stored in the fridge at 4-8 ºC.

5.2.3 Calibrators and Quality Control Preparation

AEDs were divided into 3 groups according to their proposed calibration curve ranges as explained in Table 5-1.

Table 5-1: AEDs Groups According to Their Proposed Calibration Curve Ranges.

Group 1 (0.5-50 mg/L)		Group 2 (0.05-10 mg/L)		Group 3 (5-300 mg/L)	
Drug	**Abbrev.**	**Drug**	**Abbrev.**	**Drug**	**Abbrev.**
Carbamazepine	CBZ	Oxcarbazepine	OXC	Levetiracetam	LEV
Carbamazepine epoxide	CBZO	Tiagabine	TIG	Vigabatrin	VIG
Eslicarbazepine acetate	ESL	Retigabine	RTG	Valproic acid	VPA
S-Licarbazepine	S-LE	N-acetyl retigabine	NA-RTG		
Gabapentin	GBP				
Lacosamide	LAC				
Lamotrigine	LTG				
Phenobarbital	PBT				
Phenytoin	PHT				
5-(3-Hydroxyphenyl)-5-phenylhydantoin	p-HPPH				
Pregabalin	PGR				
Rufinamide	RFM				
Stiripentol	STP				
Topiramate	TPR				
Zonisamide	ZNS				

Group 1 and 2 standards were purchased as solutions at concentration of 0.5 mg/mL for rufinamide, 0.1 mg/mL for N-acetyl retigabine and 1mg/mL for other AEDs. These standards were used as stock solutions. Levetiracetam, valproic acid and vigabatrin (group 3), were purchased as powder. A weight of 10 mg of each drug was dissolved in 1 mL methanol to obtain a stock concentration of 10 mg/mL.

Two separate set of working solutions; 4 working solutions each, were prepared at concentrations of 100 mg/L for working solutions 1, 2 and 3 and 1 g/L for working solution 4 by combining certain volumes of the stock solutions in 5 mL volumetric flask and made up to volume with methanol as detailed in Table 5-2.

Eight calibration standard solutions were prepared in methanol by combining certain volumes of the four working solutions in 5 mL volumetric flasks and made up to volume with methanol to achieve the target concentrations as detailed in Table 5-3.

Table 5-2: Preparation of Calibrators and QCs Working Solutions.

Working Solution 1: (5 mL volumetric flask)			
Analyte	**Stock Concentration**	**Amount to Add**	**Final Concentration**
CBZ	1 mg/mL	500 µL	100 mg/L
CBZO	1 mg/mL	500 µL	100 mg/L
ESL	1 mg/mL	500 µL	100 mg/L
S-LC	1 mg/mL	500 µL	100 mg/L
GBP	1 mg/mL	500 µL	100 mg/L
LAC	1 mg/mL	500 µL	100 mg/L
RFM	**0.5 mg/mL**	1000 µL	100 mg/L
TPR	1 mg/mL	500 µL	100 mg/L
Working Solution 2: (5 mL volumetric flask)			
Analyte	**Stock Concentration**	**Amount to Add**	**Final Concentration**
LTG	1 mg/mL	500 µL	100 mg/L
PGR	1 mg/mL	500 µL	100 mg/L
PHT	1 mg/mL	500 µL	100 mg/L
p-HPPH	1 mg/mL	500 µL	100 mg/L
STP	1 mg/mL	500 µL	100 mg/L
ZNS	1 mg/mL	500 µL	100 mg/L
PBT	1 mg/mL	500 µL	100 mg/L
Working Solution 3: (5 mL volumetric flask)			
Analyte	**Stock Concentration**	**Amount to Add**	**Final Concentration**
OXC	1 mg/mL	500 µL	100 mg/L
TIG	1 mg/mL	500 µL	100 mg/L
RTG	1 mg/mL	500 µL	100 mg/L
NA-RTG	**0.1 mg/mL**	1000 µL	100 mg/L
Working Solution 4: (5 mL volumetric flask)			
Analyte	**Stock Concentration**	**Amount to Add**	**Final Concentration**
LEV	10 mg/mL	500 µL	1 g/L
VIG	10 mg/mL	500 µL	1 g/L
VPA	10 mg/mL	500 µL	1 g/L

Table 5-3: Preparation of Calibrators and QCs Working Solutions.

Volumetric Flask (5 mL) No.	Calibrator No.	Amount Added (µL)				Final Concentration (mg/L)		
		Working Solution 1	Working Solution 2	Working Solution 3	Working Solution 4	Group 1	Group 2	Group 3
1	Cal 1	25	25	2.5	25	0.5	0.05	5
2	Cal 2	50	50	5	50	1	0.1	10
3	Cal 3	125	125	12.5	125	2.5	0.25	25
4	Cal 4	250	250	25	250	5	0.5	50
5	Cal 5	500	500	50	375	10	1	75
6	Cal 6	1000	1000	125	500	20	2.5	100
7	Cal 7	1750	1750	250	1000	35	5	200
8	Cal 8	2500	2500	500	1500	50	10	300

Three QC samples (low, medium and high) were directly made in whole blood, plasma and serum using the second set of working solutions by combining certain volumes of the four working solutions in 10 mL volumetric flasks. Then, the methanol solvent was evaporated under nitrogen at 25ºC to avoid blood precipitation. The residue was finally reconstituted with 10 mL whole blood, plasma or serum to achieve the target concentrations as detailed in Table 5-4. QC concentrations were 3, 20 and 40 mg/L for group 1; 2, 4 and 9 mg/L for group 2; and 20, 120 and 200 mg/L for group 3. For each QC, 0.5 mL aliquots were placed into labelled 1.5 mL polystyrene screw cap tubes. All QCs and stock solutions were stored at -20°C, and working solutions were stored at 4°C.

Table 5-4: Preparation of QCs in Whole Blood, Plasma and Serum.

Volumetric Flask (10 mL) No.	QC No.	Amount Added (µL)				Final Concentration (mg/L)		
		Working Solution 1	Working Solution 3	Working Solution 4	Working Solution 5	Group 1	Group 2	Group 3
1	QC 1	300	300	200	200	3	2	20
2	QC 2	2000	2000	400	1200	20	4	120
3	QC 3	4000	4000	900	2000	40	9	200

5.2.3.1 Preparation of Internal Standards

Although it is advisable to use deuterated internal standards for quantitive analysis, however, due to the high cost of the analytes included in this method and their deuterated internal standards, it was decided to use analog internal standards as alternatives. CBZ-DiOH was used for carbamazepine and its derivatives, GBP-D_{10} was used for GBP, PGR and VIG and TUB, an anti-diabetic medication that is rarely used in combination with antiepileptic drugs was used for the rest analytes. Three internal standards, GBP-D_{10}, TUB and CBZ-DiOH were used. GBP-D_{10} was readily purchased in methanol at concentration of 100 mg/L. TUB and CBZ-DiOH were purchased as a powder. An amount of 1 mg was dissolved in 10 mL methanol to obtain a 100 mg/L solution of TUB and CBZ-DiOH. Using a 100 mL volumetric flask, a combined internal standard solution was prepared at 5 mg/L and made up to volume with methanol as outlined in Table 5-5 below.

Table 5-5: Internal Standard Solution Preparation.

Internal Standard	Abbrev.	Stock Solution (mg/L)	Amount added (mL)	Final Concentration (mg/L)
Gabapentin-D_{10}	GBP-D_{10}	100	0.5	5
Tolbutamide	TUB	100	0.5	5
10,11 Dihydro carbamazepine	CBZ-DiOH	100	0.5	5

5.2.4 Calibration Curve Preparation

Calibration curves were extracted in duplicate by adding the following volumes to 100 µL of blank matrix (whole blood, serum or plasma): 100µL of the standards, 100 µL of the internal standard solution and 200 µL of methanol (methanol total volume 400 µL). The standards were vortex mixed for 30 seconds and centrifuged for 10 minutes at 10000 rpm. An aliquot of 200 µL of the supernatant was transferred to a LC vial and diluted with 1.3 mL of deionized water. A 5 µL aliquot of the diluted supernatant was injected and analysed by LC/MS/MS.

5.2.5 Sample Preparation

The same protein precipitation extraction used at FMS was applied. A 100 µL aliquot of the sample (blood, plasma or serum) was transferred to a 2-mL snap top polypropylene micro-centrifuge tube. To this, 100 µL of combined internal standard solution and 300 µL of methanol were added (methanol total volume 400 µL). The standards were vortex mixed for 30 seconds and centrifuged for 10 minutes at 10000 rpm. An aliquot of 200 µL of the supernatant was transferred to a LC vial and diluted with 1.3 mL of demonized water. A 5 µL aliquot of the diluted supernatant was injected and analysed by LC/MS/MS.

5.2.6 Optimisation of Chromatographic Conditions

In order to optimize the fragmentor voltage and the collision energy required to analyse the drugs on LC/MS/MS, the stock solutions were diluted 1:100 to obtain one working solution for each drug at 10 mg/L. These were achieved by adding 100 µL of the 1g/L stock solution to a 10 mL volumetric flask and made up to volume with mobile phase mixture (2 mM ammonium Acetate/ MeOH: 50/50). For LEV, VPA and VIG, a 10 µL of the 10g/L stock solution to a 10 mL volumetric flask and made up to volume with mobile phase mixture.

Post column infusion was carried out using a 500µl syringe (from COSAGE, Australia) which operated at a flow rate of 10 µL/min. All the drugs were re-tuned and their precursor ion, fragmentor voltage, collision energy, and product ions were recorded in order to attain a high sensitivity. The response was monitored visually and results were recorded on an excel spreadsheet.

5.2.7 Instrumentation

An Agilent LC/MS/MS triple quadruple G6430A mass spectrometer equipped with Agilent 1200 series auto sampler, quaternary pump SL with degasser and thermostatted column compartment was used. This system is very similar to G6420A used in the previous project. The only difference is that G6430A has a second turbo pump added to improve the vacuum (237). Otherwise, they both operate in the same way and use the same version of Agilent Mass-Hunter Work station (version: B.01.05). Electrospray ionization (ESI) was used.

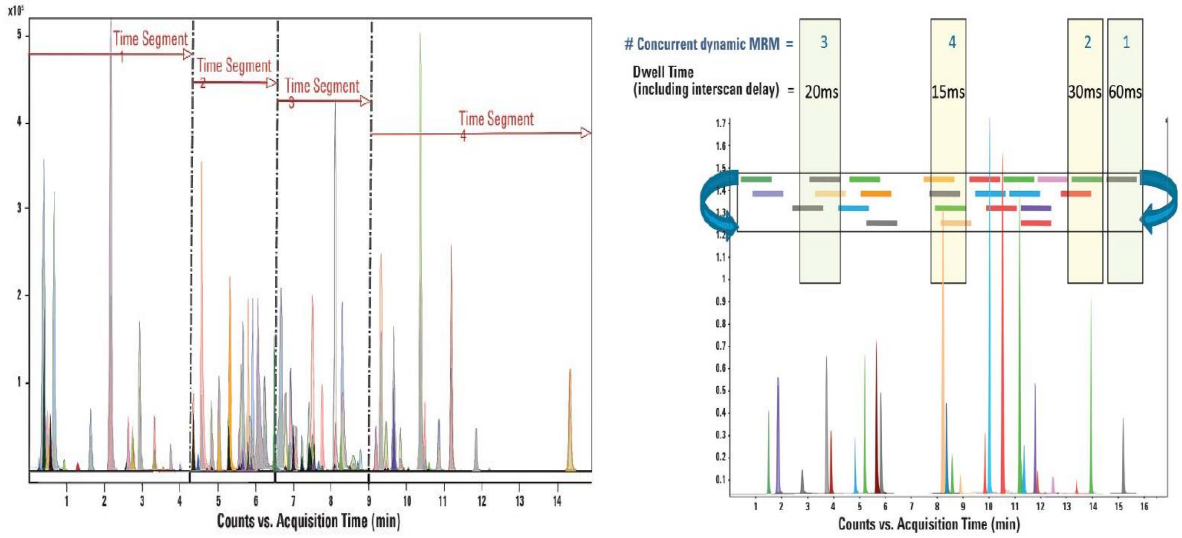
The optimal conditions were achieved using a nebulizer pressure at 15 psi, a capillary voltage of 4000 V, nitrogen gas heated to 300 °C and delivered at 10 mL/min. The column used was a Phenomenex Gemini C18 (150 mm x 2.0 mm, 5µm) coupled with a C18 guard column (4.0 mm x 2.0 mm). The column temperature was maintained at 40 °C. Gradient elution was employed using a mobile phase consisting of A: 2 mM ammonium acetate in water and B: 2 mM ammonium acetate in methanol at a flow rate of 0.3 mL/min. The total run time was 17 minutes. The gradient mobile phase system started at 80:20 A/B increasing to 50:50 A/B within 2 minutes. This percentage was maintained for 6 minutes before being increased to 10:90 A/B for 2 minutes. The percentage was finally decreased to 80:20 A/B for 7 minutes in order to condition the column before the next injection. Data analysis was performed using Agilent Mass-Hunter Workstation.

5.2.8 MRM vs DMRM

The method was initially developed using MRM mode to determine the retention time and then DMRM was applied similar to the developed method at FMS.

The main difference between conventional MRM and DMRM is that conventional MRM uses time segments and predefined sets of MRM transitions are monitored for each segment, whereas DMRM removes the requirement to create well-defined segments because it uses the retention time window for each analyte and MRM transitions are built

up dynamically throughout the LC/MS run (Figure 5-1). With DMRM, the analyte is only monitored when it is eluting from the LC. This approach is of great benefit when analysing a large number of drugs as conventional MRM may be unable to cope with this. In case of having a large number of transitions per segment, it will be necessary to either decrease the amount of time required to analyse a single MRM transition or the so-called "Dwell time" or increase the cycle time for each MS scan. Reducing the dwell time may cause collision cell cross talk while increasing the MS cycle may decrease the number of collected data points during the elution. Both factors can affect the data quality (245).



Conventional MRM Dynamic MRM

Figure 5-1: The Difference Between MRM and DMRM Scan Mode on LC/MS/MS (245).

Another advantage of using DMRM is that it significantly improves the LOD for some drugs at very low concentrations due to the improved sampling across the chromatographic peak resulting in better peak symmetry and more accurate quantitation.

5.2.9 Method Validation

Due to the addition of 5 new drugs, 2 other matrices and the use of an Agilent 6430 mass spectrometer instead of an Agilent 6420 mass spectrometer, a re-validation was required. The method was re-validated according to the standard practices for method validation in forensic toxicology (SWGTOX, May 2013) for whole blood, serum and plasma (7). (See Chapter 3 for details).

Selectivity was assessed only in plasma/serum because it had been evaluated for whole blood previously. It was evaluated using negative case samples because free blank matrices

were not available at the time of the project. Specificity was assessed by spiking drug-free matrix with each AED individually. Interferences were examined visually.

Limits of Detection (LOD): Instrument LODs were determined using decreasing concentrations of non-extracted drug standard solutions and analysing in duplicate for three separate runs. Assay LODs were determined for each matrix (blood, plasma and serum) using 3 different source of blank matrix samples spiked with decreasing concentrations of AEDs. These were analysed in duplicate for three separate runs.

Linearity was assessed by analyzing five separate calibration curves per matrix prepared by spiking blank blood, plasma or serum with AEDs working solution at 8 concentrations ranging from 5-300 mg/L for levetiracetam, valproic acid and vigabatrin; 0.05-10 mg/L for retigabine and tigabine; 0.1-10 mg/L for N-acetyl retigabine and oxcarbzepine; 0.5-50 mg/L for carbamazepine, carbamazepine epoxide, eslicarbazepine acetate, S-licarbazepine, gabapentin, lacosamide, rufenamide, stiripentol and topiramate; 1-50 µg/mL lamotrigine, phenytoin, p-HPPH, pregabalin and zonisamide; and 2.5-50 µg/mL for phenobarbital. All calibration points were freshly prepared in duplicate in each batch over 5 different days (10 replicates per each concentrations). Calibration curves were generated by plotting the peak area ratio versus the spiked analyte concentrations and applying weighed (1/x) least-squares linear regression analysis. Blank matrix with internal standard was run with each batch but not included in the calibration curve. The correlation coefficient (R^2) was calculated. The R^2 values should be greater than 0.99.

Accuracy and precision were assessed by analysing replicates of spiked controls at 3 different concentrations (low, medium and high) in serum and plasma. In whole blood, 3 different concentrations were prepared for the new drugs, whereas only two concentrations (low and high) were used for the drugs included in the original method because they had been fully validated previously. Intra-day precision was calculated from 3 replicates per QC per matrix in one batch. Inter-day precision was determined over 5 different runs. Accuracy was expressed as a percentage of the nominal concentration and precision was established by the percentage of the co-efficient of variation (% CV).

Recoveries and Matrix Effects were evaluated using the post-extraction addition approach for the new drugs in whole blood and for all 22 AEDs in serum and plasma (see 2.2.12.4 for details) (8). Retigabine matrix effect and recovery were re-evaluated due to the validation failure of retigabine in the original method. Its recovery was 33% and it

exhibited a high matrix suppression effect with a matrix factor of 0.33 (Chapter 3, Table 3-5).

Carryover was tested by injecting three blank controls after two injections of the upper limit of quantification in the calibration curve. Carryover was evaluated by dividing the blank peak area at the expected retention time by the mean peak area of the ULOQ and multiplying by 100. No carryover is considered if the value is lower than 10%.

Stability: drug stability from sample preparation until analysis and processing is essential to ensure the accuracy and precision of the forensic analysis and interpretation. A stability study was carried out using drug free matrix (whole blood, plasma and serum). Storage conditions evaluated were:

- Room temperature (approximately 25 ºC) for 24 hours. Samples spiked in triplicate at two concentrations (low and high).

- In process stability (auto sampler) at approximately 25ºC was evaluated by re-injecting QCs at two concentrations after 72 hours. Samples were stored in the autosampler during the study duration.

- Freeze-thaw cycles stability. Samples spiked in triplicate using one concentration.

The concentrations obtained were compared with those from freshly spiked controls. The results were also compared with those obtained from the validation of the original method for whole blood.

5.3 Results and Discussion

5.3.1 Optimisation of AED Separation and Chromatographic Conditions

The optimized precursor ions, fragmentor voltages, collision energies, and product ions for the AEDs are shown in Table 5-6. The chromatograms of the AEDs are shown in Figure 5-2.

Eslicarbazepine acetate (ESL) is a chiral pro-drug which is quickly and extensively metabolized to S-licarbazepine (S-LC) (95–98%) and, to a minor extent, R-licarbazepine

(R-LC) and OXC (275). On the other hand, oxcarbazepine (OXC) is a chiral pro-drug which, in humans, is reduced to the active licarbazepine metabolite, appearing in plasma as S-LC and R-LC in approximately a 4:1 enantiomer ratio (276). As a result, the method has been validated using S-LC as a metabolite of both OXC and ESL.

Post column infusion has the advantage of giving a more accurate tune compared with the tuning (optimizer software and sample injection procedure) used in the original method because of the possibility to manually tune the compounds and adjust the peak centre and the product ion (see 2.3.1, Chapter 2 for more details). Furthermore, it is faster and more reliable for method development.

Table 5-6: Optimization of MRM transitions, Fragmentor Voltage, Collision Energy, Ionization Mode and Retention Time of 22 AEDs.

AEDs	Precursor (m/z)	Quantifier (m/z)	Qualifier (m/z)	Frag[a] (V)	CE[b] (eV)	RT[c] (mins)	Ionization mode
CBZ	237.3	194.2	192.0	140	20	12.0	+
CBZO	253.1	236.1	210.1	90	2	8.7	+
ESL	297.2	194.1	237.2	100	20	11.2	+
GBP	172.2	154.2	137.0	110	10	3.4	+
LAC	251.1	108.1	91.1	90	5	7.3	+
LEV	171.1	154.0	69.1	50	5	3.7	+
LIC/S-LC	255.1	194.1	237.2	80	20	8.6	+
LTG	256.1	166.0	211.0	180	30	7.8	+
OXC	253.2	180.1	208.1	170	30	9.4	+
PBT	231.1	188.2	85.0	100	5	8.3	-
PGR	160.2	142.2	97.1	140	10	3.4	+
PHT	251.2	102.0	208.2	110	10	11.0	-
p-HPPH	267.2	118.1	224.1	125	10	7.9	-
RFM	239.0	127.0	222.0	90	20	7.3	+
RTG	304.2	230.1	258.1	140	20	12.9	+
NA-RTG	274.3	256.0	232.1	115	10	9.3	+
STP	217.2	159.2	187.2	100	10	14.3	+
TIG	376.1	247.1	278.2	140	20	13.4	+
TPR	338.1	78.0	96.0	160	20	8.6	-
VIG	130.1	113.1	71.0	65	10	1.6	+
VPA	143.1	143.1	n/a	100	0	11.7	-
ZNS	211.2	119.1	147.1	115	10	6.5	-
Internal Standards							
CBZ-DiOH	239.1	194.1	n/a	140	20	12.8	+
GBP-D_{10}	182.1	164.3	n/a	110	10	3.3	+
TUB	269.2	170.0	n/a	120	10	11.1	-

a: Fragmentor voltage, b: collision energy, c: retention time.

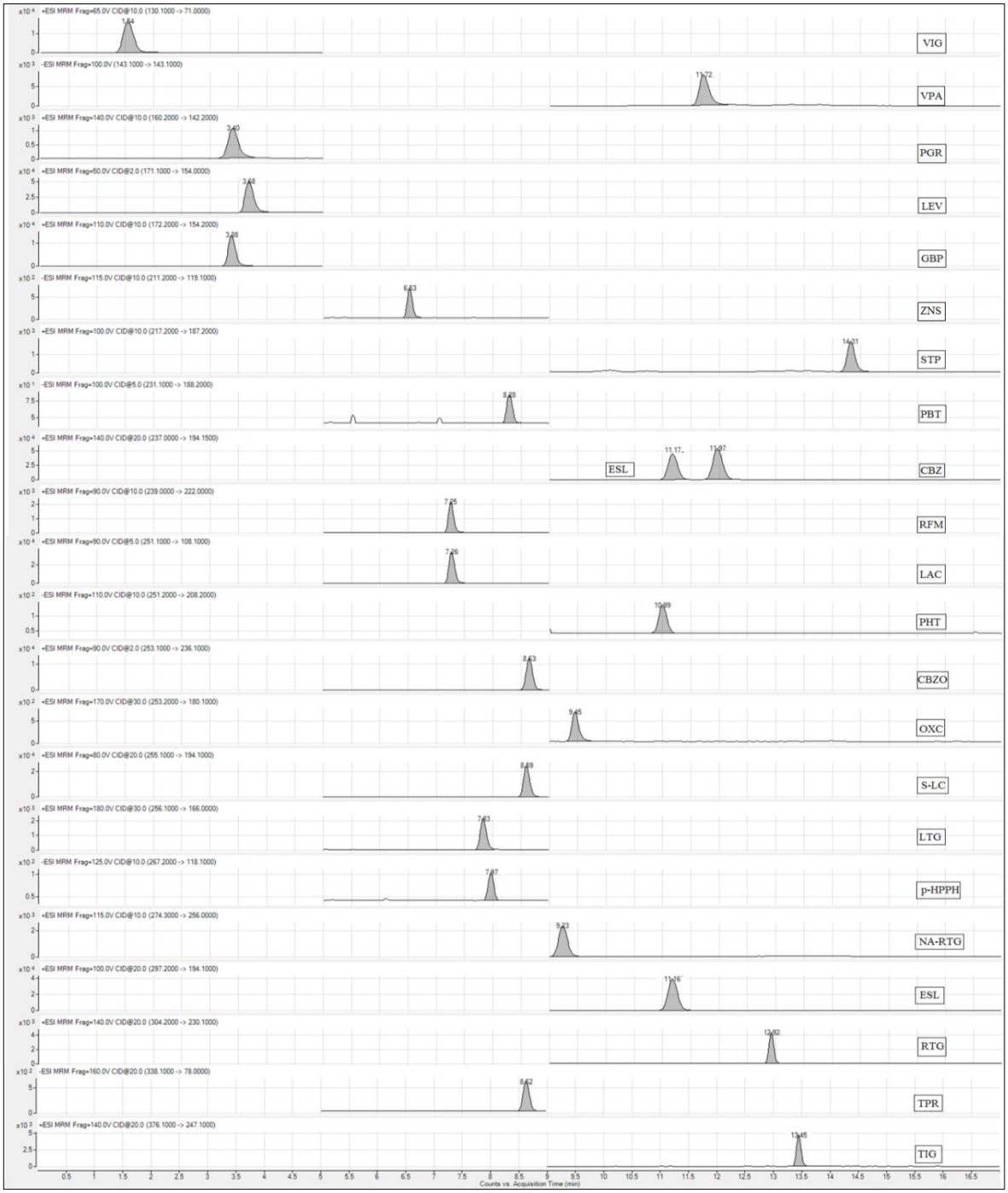


Figure 5-2: The Chromatogram of the 22 AEDs Using the Extracted Medium QC (20 mg/L for Group 1, 4 mg/L for Group 2 and 200 mg/L for Group 3) from Plasma.

Although Agilent 6420 and 6430 mass spectrometry systems are from the same series, there were some variations regarding the product ions and the collision energy for some drugs (Chapter 2, Table 2-13). For instance, with Agilent 6430, the eslicarbazepine acetate quantifier ion was 194.1 which presented the highest abundance under the same conditions and 237.2 was used as a qualifier ion whereas it was the other way round using the Agilent 6420. This was also noticed for oxcarbazepine and levetiracetam. For other drugs like phenytoin, the quantifier ion was completely different; 102 instead of 180. Some drugs had the same quantifier ion but different qualifier ion such as carbamazepine and

carbamazepine epoxide; 192 instead of 179 and 210.1 instead of 180 respectively. This variation is expected with LC/MS/MS due to the mechanism of ionization. ESI collision-induced fragmentations are often not very peak-rich, so they are often not such good "fingerprints" as electron impact ionization (EI) spectra that are used in GC/MS, hence, they can vary with instrument and conditions.

5.3.2 MRM vs DMRM

DMRM was used successfully with the original method using the Agilent 6420 mass spectrometer. However, this was not the case when the method was transferred to the Agilent 6430. Two of the internal standards, TUB and CBZ-DiOH exhibited poor sensitivity using DMRM in spite of the experiments carried out to adjust the method parameters and the gradient system to achieve the same results as the original method. Hence, the method was developed using MRM with ion mode switching instead of DMRM and the dwell time was adjusted to give good sensitivity and peak shape for all the drugs with acceptable LOQs.

5.3.3 Selectivity and Specificity

No endogenous or exogenous interference was observed and none of the AEDs or their internal standards showed any interference at the retention time of the other drugs included in the method. The generated results from plasma and serum were comparable to the whole blood selectivity study in the original method (See 3.3.1, Chapter 3). The results were consistent with those expected as the 3 matrices are from the same origin and the only difference being the red cell content and fibrinogen factor. Plasma is produced when whole blood is collected in tubes that are treated with an anticoagulant and red cells are removed by centrifugation, whereas serum is plasma without clotting agents as the whole blood is left to clot first before centrifugation.

5.3.4 Limits of Detection and Limits of Quantification

Instrument and assay LOD, LLOQ and LOQ results of 22 AEDs compared to the original method LOQ are presented in Table 5-7. Assay LODs, LLOQs and LOQs had the same values in blood, plasma and serum; whereas the instrument LODs were slightly lower than the assay LODs for ESL, S-LC, NA-RTG and VIG. Compared to the original method, the LOQs for all drugs were the same except pregabalin and oxcarbazepine which had a

slightly higher LOQ using MRM compared to DMRM (1.0 and 0.1 mg/L instead of 0.5 and 0.05 mg/L respectively) but the calibration range was still fit for purpose.

Table 5-7: Instrument and Assay LOD, LLOQ and LOQ of 22 AEDs in Whole Blood, Plasma and Serum Compared to the Original Method LOQs.

AED	Instrument LOD (mg/L)	Assay LOD (mg/L)	LLOQ (mg/L)	LOQ (mg/L)	Original LOQ (mg/L)
CBZ	0.1	0.25	0.5	0.5	0.5
CBZO	0.05	0.05	0.25	0.5	0.5
ESL	0.1	0.25	0.5	0.5	0.5
GBP	0.1	0.1	0.25	0.5	0.5
LAC	0.05	0.05	0.25	0.5	0.5
LEV	0.1	0.1	0.5	5.0	5.0
S-LC	0.05	0.1	0.25	0.5	n/a
LTG	0.25	0.25	0.5	0.5	0.5
OXC	0.05	0.05	0.1	**0.1**	**0.1**
PBT	1.0	1.0	2.5	2.5	n/a
PGR	0.5	0.5	1.0	**1.0**	**0.5**
PHT	0.5	0.5	1.0	1.0	1
p-HPPH	0.5	0.5	1.0	1.0	1
RFM	0.1	0.1	0.25	0.5	n/a
RTG	0.025	0.025	0.05	0.05	0.05
NA-RTG	0.01	0.025	0.05	0.1	n/a
STP	0.25	0.25	0.5	0.5	n/a
TIG	0.01	0.01	0.025	0.05	0.05
TPR	0.25	0.25	0.5	0.5	0.5
VIG	0.1	0.5	1.0	5.0	5.0
VPA	2.5	2.5	5.0	5.0	5.0
ZNS	0.5	0.5	1.0	1.0	1.0

5.3.5 Linearity

The same calibration models were used for whole blood, serum and plasma. They were linear over the wide range of concentrations tested with an R^2 greater than 0.99 using a weighting of 1/X for all drugs in whole blood, serum and plasma except for CBZ, CBZO, ESL, LAC, LEV, LTG and VIG where quadratic regressions were used with a weighting of 1/X (Table 5-8). Seven drugs used quadratic regression in the transferred method compared to only lamotrigine in the original method, however, using this model, regression did not affect the validation process and the results were reproducible.

Non-linear calibrations over wide ranges are more common with LC/MS/MS (258, 259, 277, 278). Quadratic regression may result when the proportionality between the analyte/IS response ratio and the analyte concentration is lost due to some phenomena during sample preparation, LC analysis or MS analysis. This may lead to a bias of analyte/IS ratio resulting in non-linearity of the standard curve. The same was observed when using

different instruments or the same instrument but over a long period of time. The curve fit appeared to change from linear to quadratic due to the sensitivity variation between instruments or with time on the same instrument (279). Another reason for a non-linear calibration curve is the use of analog internal standards instead of deuterated ones. Deuterated internal standards usually compensate for variability during sample preparation and analysis process and minimize the matrix effect. However, quadratic calibrations using deuterated internal standards have also been reported (279). In such cases, non-linear ranges are caused by saturation at high concentrations during ionization or by detector saturation (280, 281). Nevertheless, using quadratic calibration curves, extends the dynamic range of the standard curves and allows a broader concentration range to be used that can save time, consumables, and labour by avoiding sample dilution.

Retigabine passed the validation with the transferred method even although the same mobile phase, column and LC/MS conditions used in the original method were applied. One reason for this would be the high pump pressure that occurred during the first project which may have affected the late elution of retigabine towards the end of the method (retention time: 15 minutes).

Table 5-8: Calibration Model and Linearity Results in Whole Blood, Plasma and Serum.

AED	Calibration Range (mg/L)	Internal Standard	Calibration Model	Whole blood R^2 (*n=5*)	Plasma R^2 (*n=5*)	Serum R^2 (*n=5*)
CBZ	0.5-50	CBZ-DiOH	Quadratic	0.999	0.999	0.999
CBZO	0.5-50	CBZ-DiOH	Quadratic	0.999	0.998	0.998
ESL	0.5-50	CBZ-DiOH	Quadratic	0.999	0.996	0.999
GBP	0.5-50	GBP-D10	Linear	0.998	0.998	0.999
LAC	0.5-50	GBP-D10	Quadratic	0.999	0.997	0.998
LEV	5.0-300	GBP-D10	Quadratic	0.999	0.999	0.999
S-LC	0.5-50	CBZ-DiOH	Linear	0.9997	0.999	0.998
LTG	1.0-50	GBP-D10	Quadratic	0.9995	0.997	0.998
OXC	0.1-10	CBZ-DiOH	Linear	0.999	0.999	0.999
PBT	2.5-50	TUB	Linear	0.999	0.995	0.998
PGR	1.0-50	GBP-D10	Linear	0.997	0.999	0.998
PHT	1.0-50	TUB	Linear	0.9997	0.996	0.998
p-HPPH	1.0-50	TUB	Linear	0.998	0.999	0.999
RFM	0.5-50	GBP-D10	Linear	0.999	0.996	0.996
RTG	0.05-10	CBZ-DiOH	Linear	0.999	0.998	0.997
NA-RTG	0.1-10	CBZ-DiOH	Linear	0.996	0.996	0.997
STP	0.5-50	TUB	Linear	0.994	0.998	0.998
TIG	0.05-10	CBZ-DiOH	Linear	0.998	0.997	0.997
TPR	0.5-50	TUB	Linear	0.997	0.998	0.999
VIG	5.0-300	GBP-D10	Quadratic	0.999	0.998	0.999
VPA	5.0-300	TUB	Linear	0.997	0.999	0.999
ZNS	1.0-50	TUB	Linear	0.996	0.999	0.999

5.3.6 Accuracy and Precision

Accuracy and precision results are presented in Table 5-9, Table 5-10 and Table 5-11for whole blood, plasma and serum respectively. As mentioned previously, precision and accuracy for AEDs included in the original method were assessed using two concentrations while serum and plasma were assessed using three concentrations. The intraday precision values were < 9.0% for whole blood, < 10% for plasma (except PBT where the medium QC was 16.2% in plasma) and < 9.0% for serum. The inter-day precision values were < 14.4% for whole blood, < 13.1% for plasma and serum. The accuracy values were within the acceptable range of ±15% of the nominal concentrations. The intra-day accuracy was between 91.4-113% for whole blood, 89.7-110.6% for plasma and between 85.7-113.2% for serum. The inter-day accuracy was between 90.9-113% for whole blood, 91.7-110.6% for plasma and between 91.8-111.8% for serum.

Table 5-9: Intra- and Inter-day Precision and Accuracy Results of Whole Blood Using 3 Different QCs at Different Concentrations of 3, 20 and 40 mg/L for Group 1; 2, 4 and 9 mg/L for Group 2; and 20, 120 and 200 mg/L for Group 3.

AED	Precision						Accuracy					
	Intra-day (%) (n=3)			Inter-day (%) (n=15)			Intra-day (%) (n=3)			Inter-day (%) (n=15)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
CBZ	5.0	n/a	6.4	9.1	n/a	7.6	100.4	n/a	99.9	100.4	n/a	99.9
CBZO	2.9	n/a	4.7	6.7	n/a	13.1	103.4	n/a	112.4	103.4	n/a	93.7
ESL	5.2	n/a	5.8	12.6	n/a	14.4	91.4	n/a	94.5	91.4	n/a	110.2
GBP	4.0	n/a	5.7	9.1	n/a	4.3	99.9	n/a	102.2	94.9	n/a	102.0
LAC	3.1	n/a	4.2	7.5	n/a	15.9	109.0	n/a	93.3	109.0	n/a	93.3
LEV	4.4	n/a	3.6	8.3	n/a	10.4	107.8	n/a	96.0	107.8	n/a	96.0
LIC/S-LC	2.2	4.8	6.6	5.8	5.4	7.4	105.9	108.4	101.3	105.9	108.4	101.3
LTG	7.9	n/a	8.2	11.7	n/a	14.2	97.5	n/a	101.8	97.5	n/a	100.3
OXC	5.5	n/a	7.5	7.1	n/a	11.1	95.4	n/a	112.1	95.4	n/a	112.1
PBT	4.1	5.9	9.0	2.9	12.9	5.2	98.8	98.3	103.0	94.9	98.3	103.0
PGR	6.5	n/a	5.9	13.9	n/a	13.6	95.7	n/a	99.5	104.2	n/a	99.5
PHT	8.1	n/a	8.8	9.7	n/a	3.8	100.1	n/a	103.9	100.1	n/a	90.9
p-HPPH	7.0	n/a	5.2	8.4	n/a	11.3	97.6	n/a	95.0	97.6	n/a	95.0
RFM	2.4	4.1	3.2	3.7	9.7	3.5	108.7	106.0	96.4	108.7	106.0	96.4
RTG	6.4	6.6	6.5	4.7	6.7	8	106.8	106.1	101.8	101.8	106.8	106.1
NA-RTG	7.8	5.5	5.7	12.7	8.8	7.0	98.5	100.5	101.7	98.5	100.5	101.7
STP	5.9	6.2	7.1	4.6	9.6	3.5	106.5	98.7	101.4	106.5	98.7	101.5
TIG	5.2	n/a	2.9	14.0	n/a	9.2	113.0	n/a	97.6	113.0	n/a	97.6
TPR	4.9	n/a	5.2	6.9	n/a	5.3	102.3	n/a	98.5	102.3	n/a	98.5
VIG	4.7	n/a	4.1	9.0	n/a	7.8	99.8	n/a	99.6	99.8	n/a	99.6
VPA	4.5	n/a	5.6	8.4	n/a	5.9	111.1	n/a	101.5	105.8	n/a	101.5
ZNS	2.9	n/a	5.4	6.7	n/a	4.7	103.4	n/a	94.7	103.4	n/a	94.7

Table 5-10: Intra- and Inter-day Precision and Accuracy Results of Plasma Using 3 Different QCs at Different Concentrations of 3, 20 and 40 mg/L for Group 1; 2, 4 and 9 mg/L for Group 2; and 20, 120 and 200 mg/L for Group 3.

AED	Precision						Accuracy					
	Intra-day (%) (n=3)			Inter-day (%) (n=15)			Intra-day (%) (n=3)			Inter-day (%) (n=15)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
CBZ	1.1	2.2	1.6	1	0.2	4	97.9	101.3	102.5	97.9	101.3	102.5
CBZO	3.2	0.9	1.9	4.9	3.3	1.6	101.0	104.6	101.1	101.0	104.6	101.1
ESL	1.8	4.9	1.8	2.4	4.3	5.4	99.9	93.4	99.5	99.9	106.7	99.5
GBP	1.4	2.6	0.8	0.8	2.7	3.5	96.2	98.3	104.6	105.8	98.3	104.6
LAC	1.5	1.7	1.3	1.9	3.6	4.5	99.2	100.1	100.7	99.2	100.1	100.7
LEV	2.6	1.4	3.6	2.1	4.2	2.9	96.5	96.3	97.3	96.5	96.3	97.3
LIC	1.4	1.3	4.3	5.4	5.6	0.7	101.1	101.5	91.7	101.1	101.5	91.7
LTG	2.8	2.6	3.1	6.5	10.3	10.7	110.6	103.3	99.9	110.6	103.3	103.1
OXC	2.3	4.3	2.7	4.6	1.6	3.7	95.2	107.7	100.2	95.2	107.7	100.2
PBT	5.0	16.2	6.0	7.9	4.7	3.9	93.7	92.2	105.0	93.7	92.2	91.9
PHT	10.0	6.1	4.6	2.0	3.9	3.8	96.6	103.8	101.8	106.3	103.8	101.8
PGR	8.7	3.6	2.0	4.3	12.0	12.0	102.1	98.0	105.0	102.1	98.0	107.0
HPPH	9.9	7.4	4.5	2.4	10.9	2.8	95.4	100.2	97.9	95.4	100.2	97.9
RFM	3.3	1.3	0.9	13.1	4.2	0.2	107.1	104.0	102.5	107.1	104.0	102.5
RTG	2.6	6.9	2.1	0.8	6.5	8.2	89.6	106.5	96.2	99.1	106.5	100
NA-RTG	2.3	2.6	2.7	2.8	4.7	4.8	107.8	103.4	102.8	107.8	103.4	102.8
STP	5.0	3.8	3.2	2.8	9.9	6	98.5	108.7	103.8	103.8	100.1	98.5
TIG	3.2	2.2	1.0	9.1	8	9.2	102.5	108.8	96.1	102.5	102.9	96.6
TPR	7.6	3.7	3.3	7.6	2.0	1.5	103.9	103.3	103.1	103.9	103.3	103.1
VIG	2.6	4.9	1.4	5.6	2.6	1.7	98.5	97.3	98.9	98.5	97.3	98.9
VPA	3.5	3.0	2.7	4.3	3.9	0.7	95.0	101.4	104.0	95.0	101.4	104.0
ZNS	8.7	7.2	3.1	7.3	8.6	3.9	105.6	98.9	108.5	105.6	98.9	95.0

Table 5-11: Intra- and Inter-day Precision and Accuracy Results of Serum Using 3 Different QCs at Different Concentrations of 3, 20 and 40 mg/L for Group 1; 2, 4 and 9 mg/L for Group 2; and 20, 120 and 200 mg/L for Group 3.

AED	Precision						Accuracy					
	Intra-day (%) (n=3)			Inter-day (%) (n=15)			Intra-day (%) (n=3)			Inter-day (%) (n=15)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
CBZ	2.4	1.9	1.1	1.0	0.7	2.6	98.2	98.3	99.9	98.2	98.3	99.9
CBZO	2.8	0.9	2.9	4.8	0.4	0.5	94.1	103.6	95.4	94.1	103.6	95.4
ESL	2.2	3.6	1.9	5.4	5.0	7.7	93.2	85.7	96.8	93.2	97.9	96.8
GBP	2.2	2.5	2.0	1.8	3.9	0.6	96.7	96.3	103.3	106.3	96.3	103.3
LAC	1.9	2.7	3.7	4.2	1.1	2.7	96.2	96.3	100.7	96.2	96.3	100.7
LEV	1.8	2.2	0.8	0.6	0.9	3.7	94.3	96.7	94.2	94.3	96.7	94.2
LIC	1.1	1.8	2.3	1.7	2.0	2.1	106.2	107.3	104.8	106.2	107.3	104.8
LTG	1.2	1.6	2.3	3.7	2.6	4.8	107.9	94.6	92.1	107.9	94.6	92.1
OXC	2.0	2.7	2.8	4.6	1.0	4.7	95.3	102.9	97.8	95.3	102.9	97.8
PBT	3.1	8.0	4.3	4.0	10.0	2.8	107.7	102.0	97.4	107.0	101.0	97.4
PHT	4.6	9.0	3.3	5.5	9.6	4.9	105.9	101.9	97.9	105.9	101.9	97.9
PGR	5.5	3.1	2.9	2.2	4.3	5.7	94.3	101.5	113.2	94.3	96.9	109.8
HPPH	1.6	8.7	4.2	7.7	5.7	5.7	101.4	102.8	99.4	101.4	102.8	99.4
RFM	1.8	3.0	1.7	7.2	1.2	3.4	105.3	107.7	102.1	105.3	107.7	102.1
RTG	2.1	1.3	2.6	2.2	0.5	7.6	103.0	110.5	97.6	103.0	110.5	97.6
NA-RTG	1.9	2.5	1.6	4.5	1.6	3.8	107.5	98.8	100.2	107.5	98.8	100.2
STP	1.2	4.4	2.9	6.8	7.6	2.9	104.1	98.6	106.4	104.1	110.9	106.4
TIG	1.6	0.7	4.3	7.7	6.2	5.7	111.8	92.3	103.2	111.8	92.3	91.8
TPR	4.0	4.6	4.7	3.3	4.0	3.3	106.0	102.1	100.8	106.0	102.1	100.8
VIG	1.2	3.1	1.9	2.0	6.6	7.8	93.0	93.8	96.8	93.0	93.8	96.8
VPA	4.8	4.6	4.3	0.6	3.2	2.4	94.0	102.7	107.6	94.0	102.7	107.6
ZNS	3.1	3.6	6.1	2.0	13.1	5.3	101.8	104.2	112.5	101.8	104.2	98.4

5.3.7 Matrix Factor and Recovery

The matrix factor and recovery results of 2 QCs (low and high) using 6 different sources of matrix are detailed in Table 5-12 and Table 5-13 for the 22AEDs in plasma and serum respectively and

Table 5-14 for new drugs added in whole blood. Matrix factor values were within the acceptable range for all the drugs (within ± 1.25) with standard deviation less than 20%. Recovery was greater than 79% for all the AEDs in the three matrices except RTG and its metabolite NA-RTG which were about 70% in whole blood compared to their recoveries from serum and plasma which were greater than 93%. The recovery and matrix effect results in blood were comparable to the values obtained in the original method except for

retigabine (> 71%). Its recovery and matrix factor in the original method were 33% and 0.33 respectively. Interestingly, retigabine exhibited better recovery and acceptable matrix suppression effect (MF = 0.85) in the transferred method.

Table 5-12: Recovery and Matrix Factor Values for 22 AEDs Using Low and High QCs (3 and 40 mg/L for Group 1; 2, 9 mg/L for Group 2; and 20 and 200 mg/L for Group 3) in Plasma (n=6 per QC).

AEDs	QC1		QC2	
	Recovery (%)	*Matrix Factor*	*Recovery (%)*	*Matrix Factor*
CBZ	102	1.06±0.01	98	1.10±0.04
CBZO	101	1.07±0.02	99	1.08±0.04
ESL	99	1.04±0.02	95	1.06±0.05
GBP	101	1.04±0.02	98	1.13±0.06
LAC	104	1.09±0.01	99	1.10±0.03
LEV	104	1.13±0.03	97	1.06±0.04
S-LC	105	1.11±0.01	102	1.12±0.03
LTG	86	1.2±0.04	105	1.14±0.2
OXC	109	1.03±0.06	96	1.03±0.05
PBT	79	1.24±0.17	99	1.05±0.07
PGR	104	1.05±0.08	97	1.15±0.06
PHT	95	1.10±0.14	98	1.06±0.08
p-HPPH	109	1.07±0.04	99	1.04±0.08
RFM	104	1.07±0.04	98	1.09±0.06
RTG	107	0.88±0.02	93	0.94±0.05
NA-RTG	103	0.99±0.03	100	1.07±0.05
STP	100	1.15±0.03	99	1.06±0.05
TIG	94	0.93±0.17	102	1.09±0.11
TPR	98	1.18±0.03	98	1.07±0.05
VIG	100	1.11±0.03	93	1.12±0.06
VPA	105	1.06±0.07	102	1.04±0.05
ZNS	104	1.08±0.13	98	1.06±0.05

Table 5-13: Recovery and Matrix Factor Values for 22 AEDs Using Low and High QCs (3 and 40 mg/L for Group 1; 2, 9 mg/L for Group 2; and 20 and 200 mg/L for Group 3) in Serum (n=6 per QC).

AEDs	QC1		QC2	
	Recovery (%)	*Matrix Factor*	*Recovery (%)*	*Matrix Factor*
CBZ	104	1.04±0.01	103	1.04±0.08
CBZO	103	1.04±0.02	102	1.03±0.05
ESL	104	1.0±0.02	102	0.99±0.06
GBP	106	0.99±0.02	103	1.03±0.06
LAC	104	1.07±0.01	103	1.04±0.05
LEV	105	1.10±0.01	105	0.99±0.08
S-LC	104	1.09±0.01	105	1.09±0.08
LTG	102	0.98±0.05	104	1.16±0.09
OXC	105	1.0±0.06	110	0.91±0.06
PBT	98	1.07±0.19	108	0.96±0.01
PGR	109	1.04±0.05	104	1.06±0.06
PHT	104	1.02±0.07	105	1.01±0.06
p-HPPH	109	1.06±0.05	102	0.97±0.08
RFM	104	1.06±0.01	103	1.03±0.07
RTG	107	0.96±0.03	112	0.89±0.07
NA-RTG	106	0.98±0.01	103	1.02±0.08
STP	102	1.15±0.05	105	1.02±0.09
TIG	108	0.98±0.10	103	1.04±0.05
TPR	105	1.14±0.04	107	0.98±0.07
VIG	102	0.77±0.01	101	0.83±0.06
VPA	103	1.07±0.12	108	0.98±0.08
ZNS	108	1.08±0.14	107	1.0±0.08

Table 5-14: Recovery and Matrix Factor Values for 7 Added AEDs Using Low and High QCs (3 and 40 mg/L for Group 1; 2, 9 mg/L for Group 2; and 20 and 200 mg/L for Group 3) in Whole Blood (n=6 per QC).

AEDs	QC1		QC2	
	Recovery (%)	*Matrix Factor*	*Recovery (%)*	*Matrix Factor*
S-LC	108	1.04±0.06	106	0.99±0.01
PBT	108	0.93±0.24	107	1±0.10
RFM	106	1.07±0.06	107	1.0±0.03
RTG	73	0.85±0.05	71	0.83±0.04
NA-RTG	66	0.89±0.06	68	0.88±0.02
STP	109	1.10±0.06	109	1.0±0.03

5.3.8 Carryover

No carry over was observed in the blank samples after two injections of the highest standards for all 22 AEDs in whole blood, plasma and serum. Carryover percentage after first blank injection was lower than 0.2% for all drugs. Summary of the carryover results is detailed in Table 5-15.

Table 5-15: Carryover Results of 22 AEDs After Double Injections of Extracted ULOQ.

AED	Peak Area				Carryover (%)*
	Mean ULOQ (n=2)	Blank 1	Blank 2	Blank 3	
CBZ	926727.8	7.1	0.0	0.0	**0.0**
CBZO	126984.6	2.5	0.0	0.0	**0.0**
ESL	640708.1	381.1	22.4	133.6	**0.1**
GBP	193329.0	53.6	26.6	24.9	**0.0**
LAC	344197.0	28.4	32.6	48.5	**0.0**
LEV	1119556.1	90.8	0.0	0.5	**0.0**
LIC/SLE	283631.2	196.0	26.2	60.5	**0.1**
LTG	55358.7	0.0	0.0	0.0	**0.0**
OXC	6914.8	0.0	0.0	0.0	**0.0**
PBT	4667.8	0.0	0.0	0.0	**0.0**
PGR	15696.4	1.9	0.0	0.0	**0.0**
PHT	1622.0	0.0	0.0	0.0	**0.0**
HPPH	1285.6	0.0	0.0	0.0	**0.0**
RTG	39308.8	1.4	0.0	0.0	**0.0**
NA-RTG	48539.7	108.8	7.1	0.0	**0.2**
RFM	190504.3	122.7	32.2	27.2	**0.1**
STP	30858.0	0.0	0.0	0.0	**0.0**
TIG	64803.6	0.0	0.1	0.1	**0.0**
TPR	7355.7	0.0	0.0	0.0	**0.0**
VIG	433236.8	10.6	34.3	32.0	**0.0**
VPA	201820.4	0.0	0.0	0.0	**0.0**
ZNS	7111.5	0.0	0.0	0.0	**0.0**

* Carryover percentage was calculated by dividing blank 1 peak area by ULOQ Mean peak area and multiplying by 100.

5.3.9 Stability

5.3.9.1 Bench Top and Freeze-Thaw Stability

The stability study showed that all the drugs were stable in whole blood, serum and plasma at room temperature (approximately 25ºC) for up to 24 hours (bench top stability) except

ESL, OXC , RTG and NA-RTG which showed a loss of about 70%, 20%, 21% and 36% respectively of their nominal concentrations in the three matrices. Interestingly, valproic acid exhibited *instability* in this study and it lost about 50% of its nominal concentration in the 3 matrices, whereas it showed a good stability in the original method. The variation in the results may be due to different room temperatures and the variation between geographical areas and lab atmosphere where the tests were carried out. Hence, bench top stability should be evaluated as part of any method validation and cannot rely on information published in the literature. Regarding, freeze-thaw stability, all AEDs including RTG, NA-RTG, ESL and OXC were stable after 3 freeze-thaw cycles at - 20 ºC in all three matrices.

5.3.9.2 In Process Stability

Autosampler stability was evaluated by re-injecting extracted QCs spiked in plasma in triplicate. Samples were stored on the autosampler until the next analysis. All AED extracted samples were stable in the autosampler (approximately 25ºC) for up to 72 hours. While RTG, NA-RTG, ESL and OXC were not stable in whole blood, their stability was acceptable after extraction and reconstitution in 13% methanol in water.

A summary of all bench top, freeze-thaw and autosampler stability study values for whole blood, plasma and serum is detailed in Table 5-16, Table 5-17, Table 5-18 and Table 5-19.

Table 5-16: Bench Top and Freeze-Thaw Stability Results of 22 AEDs in Whole Blood at Concentrations of 3 and 40 mg/L for Group 1; 2, 9 mg/L for Group 2; and 20 and 200 mg/L for Group 3.

AED	Bench Top Stability						Freeze-Thaw Stability		
	QC1 (mg/L, n=3)			QC2 (mg/L, n=3)			QC (mg/L, n=3)		
	T=0*	T=24	R (%)	T=0	T=24	R (%)	T=0	C3**	R (%)
CBZ	8.1	6.5	80.2	40.5	32.3	79.8	1.8	2.0	111.1
CBZO	8.3	7.9	95.2	29.7	24.4	82.2	6.5	7.3	112.3
ESL	7.5	1.6	20.7	33.8	11.8	34.8	1.5	1.2	80.0
GBP	7.1	6.8	95.8	37.0	37.5	101.4	1.8	2.1	116.7
LAC	9.3	7.5	80.6	41.6	37.5	90.1	1.9	1.8	94.7
LEV	46.9	33.1	70.6	153.6	123.6	80.5	175.7	140.1	79.7
LIC/SLE	9.2	11.2	121.7	33.6	41.4	123.2	2.2	2.4	109.1
LTG	8.5	7.9	92.9	36.5	34.0	93.2	1.4	1.3	92.9
OXC	2.2	1.8	81.8	9.3	7.9	84.9	4.6	4.1	89.1
PBT	8.3	10.1	121.7	38.7	43.3	111.9	2.2	2.1	95.5
PGR	8.6	10.3	119.8	47.5	45.6	96.0	2.5	2.4	96.0
PHT	8.3	7.9	95.2	36.6	38.6	105.5	3.6	4.0	111.1
HPPH	8.6	8.7	101.2	38.6	39.4	102.1	2.7	2.4	88.9
RTG	2.4	1.9	79.2	9.9	7.9	79.8	2.7	2.1	77.8
NA-RTG	2.6	1.6	61.5	5.3	3.4	64.2	0.6	0.5	84.7
RFM	10.3	19.5	189.3	9.4	18.1	192.6	4.4	4.6	104.5
STP	8.8	6.4	72.7	42.7	38.8	90.9	1.3	1.5	115.4
TIG	2.4	2.4	100.0	7.7	7.4	96.5	3.9	3.4	87.2
TPR	8.2	7.1	86.6	35.3	32.6	92.4	1.7	1.9	111.8
VIG	50.2	37.4	74.5	162.3	135.9	83.7	13.1	15.4	117.6
VPA	46.9	15.4	32.8	158.7	94.6	59.6	21.8	24.6	112.8
ZNS	8.9	8.0	89.7	39.0	41.8	107.2	4.3	5.1	118.6

* T represents time in hours. **C3: concentration values after third freeze-thaw cycle

Table 5-17: Bench Top and Freeze-Thaw Stability Results of 22 AEDs in Plasma at Concentrations of 3 and 40 mg/L for Group 1; 2, 9 mg/L for Group 2; and 20 and 200 mg/L for Group 3.

AED	Bench Top Stability						Freeze-Thaw Stability		
	QC1 (mg/L, n=3)			QC2 (mg/L, n=3)			QC 2 (mg/L, n=3)		
	T=0*	T=24	R (%)	T=0	T=24	R (%)	T=0	C3**	R (%)
CBZ	8.1	7.5	92.6	43.0	37.2	86.6	3.5	4.1	117.1
CBZO	7.7	6.9	89.3	30.0	25.6	85.3	13.0	15.2	116.7
ESL	7.2	5.2	72.2	33.0	26.3	79.6	2.9	3.2	110.7
GBP	6.9	6.3	91.1	35.2	37.2	105.6	3.6	4.0	111.5
LAC	8.6	7.9	91.9	42.7	39.1	91.5	4.1	4.8	117.1
LEV	44.6	39.2	87.9	149.3	129.2	86.6	176.7	182.1	103.1
LIC/SLE	7.7	8.4	108.2	32.4	28.2	87.1	3.4	3.8	111.5
LTG	7.3	7.4	101.8	28.1	30.4	108.1	2.6	3.0	115.4
OXC	2.1	1.7	79.3	9.2	6.5	71.1	5.2	6.1	117.3
PBT	7.0	6.2	88.6	35.5	30.2	85.1	3.1	3.6	118.7
PGR	6.1	5.9	96.2	30.6	29.6	96.8	4.4	3.5	79.5
PHT	8.0	7.6	95.3	38.6	30.9	80.1	5.5	5.4	98.2
HPPH	7.3	6.3	86.5	39.9	35.9	90.0	3.4	4.0	117.6
RTG	2.0	1.5	74.6	7.4	5.4	73.0	4.0	4.9	121.6
NA-RTG	2.2	1.6	73.4	4.3	3.1	72.1	0.9	1.1	122.2
RFM	8.9	8.3	93.3	18.4	16.6	89.9	4.5	4.4	96.9
STP	7.8	6.7	85.9	40.4	35.5	88.0	2.9	2.8	96.9
TIG	1.9	2.1	111.7	6.1	6.8	110.7	4.9	5.4	110.2
TPR	8.2	7.2	87.8	35.4	30.3	85.6	3.8	4.1	107.9
VIG	42.7	39.2	91.7	149.5	129.2	86.4	7.5	9.1	121.2
VPA	46.0	13.7	29.8	157.0	75.1	47.8	19.9	20.2	101.5
ZNS	7.4	6.7	90.0	37.6	32.3	85.8	5.4	5.8	107.4

* T represents time in hours. **C3: concentration values after third freeze-thaw cycle

Table 5-18: Bench Top and Freeze-Thaw Stability Results of 22 AEDs in Serum at Concentration of 3 and 40 mg/L for Group 1; 2, 9 mg/L for Group 2; and 20 and 200 mg/L for Group 3.

AED	Bench Top Stability						Freeze-Thaw Stability		
	QC1 (mg/L, n=3)			QC2 (mg/L, n=3)			QC2 (mg/L, n=3)		
	T=0*	T=24	R (%)	T=0	T=24	R (%)	T=0	C3**	R (%)
CBZ	7.9	7.5	94.9	41.2	35.0	85.0	5.6	6.5	116.1
CBZO	8.8	8.9	101.1	28.7	26.3	91.6	21.8	22.7	104.1
ESL	7.0	5.1	72.9	32.1	21.7	67.6	4.6	5.5	119.6
GBP	7.0	6.3	90.0	36.3	32.3	89.0	5.5	6.3	114.5
LAC	8.8	8.1	92.0	43.3	36.2	83.6	5.9	6.7	113.6
LEV	43.3	34.4	79.4	146.7	135.0	92.0	180.6	150.4	83.3
LIC/SLE	8.8	9.4	106.8	32.1	29.8	92.8	6.7	7.2	107.5
LTG	7.8	7.2	92.3	34.0	28.7	84.4	5.5	6.3	114.5
OXC	2.1	2.4	114.3	9.1	5.8	64.1	4.7	5.4	114.9
PBT	8.8	7.1	80.7	42.3	37.2	87.9	7.4	7.4	100.0
PGR	8.1	6.3	77.8	46.1	40.2	87.2	4.3	5.1	118.6
PHT	9.0	7.6	84.4	41.3	38.4	93.0	4.5	4.8	106.7
HPPH	8.7	7.5	86.2	42.4	35.5	83.7	5.2	5.4	103.8
RTG	2.2	1.7	77.3	8.6	6.0	69.8	5.0	5.1	102.4
NA-RTG	2.0	1.4	69.5	4.2	2.8	67.6	0.9	1.0	111.1
RFM	9.6	8.9	92.7	19.0	16.7	87.9	5.2	4.9	94.2
STP	9.6	8.4	87.5	44.4	39.0	87.8	3.8	4.5	117.2
TIG	2.4	2.1	87.5	8.7	7.4	85.1	4.7	4.3	91.9
TPR	8.5	7.3	85.9	36.5	32.0	87.7	5.5	6.2	112.7
VIG	45.4	42.4	93.4	158.1	138.0	87.3	10.7	11.6	108.4
VPA	45.9	22.4	48.8	166.6	82.7	49.6	20.1	22.4	111.4
ZNS	9.5	8.1	85.3	41.6	35.2	84.6	5.3	5.9	111.3

* T represents time in hours. **C3: concentration values after third freeze-thaw cycle

Table 5-19: In Process (Autosampler) Stability of 22 AEDs Using Extracted QCs in Plasma at Concentration of 3 and 40 mg/L for Group 1; 2, 9 mg/L for Group 2; and 20 and 200 mg/L for Group 3.

AED	QC1 (mg/L, n=3)			QC2 (mg/L, n=3)		
	T=0*	T=72	R (%)	T=0	T=72	R (%)
CBZ	8.1	7.7	94.8	43.0	38.5	89.6
CBZO	7.7	7.8	100.9	30.0	30.2	100.7
ESL	7.2	7.1	98.6	33.0	28.3	85.7
GBP	6.9	7.1	103.4	35.2	39.0	110.7
LAC	8.6	8.8	101.9	42.7	42.2	98.7
LEV	44.6	43.3	97.0	149.3	137.5	92.1
LIC/SLE	7.7	8.1	104.3	32.4	35.3	109.1
LTG	7.3	8.4	115.2	28.1	37.7	134.2
OXC	2.1	2.2	101.9	9.2	8.5	93.2
PBT	7.0	7.5	106.5	35.5	35.8	101.0
PGR	6.1	7.9	129.3	30.6	31.8	104.1
PHT	8.0	8.3	103.8	38.6	44.6	115.6
HPPH	7.3	8.2	112.8	39.9	40.3	101.0
RTG	2.0	1.6	80.6	7.4	8.8	118.9
NA-RTG	2.2	2.0	90.4	4.3	3.9	90.3
RFM	8.9	9.0	101.1	18.4	17.6	95.4
STP	7.8	9.2	118.1	40.4	44.2	109.5
TIG	1.9	2.1	113.3	6.1	6.8	110.7
TPR	8.2	7.7	93.9	35.4	36.0	101.6
VIG	42.7	43.4	101.6	149.5	142.9	95.6
VPA	46.0	44.8	97.5	157.0	159.9	101.9
ZNS	7.4	8.9	119.6	37.6	42.1	111.8

* T represents time in hours.

5.4 Conclusion

A simple, accurate and cost-effective LC/MS/MS method was transferred for the simultaneous quantification of 15 AEDs and 2 metabolites. The method was extended to include another 5 compounds; 3 AEDs and 2 metabolites, so a total of 22 AEDs; carbamazepine and its metabolite carbamazepine-10,11-epoxide, eslicarbazepine acetate, oxcarbazepine and their metabolite S-licarbazepine, gabapentin, lacosamide, lamotrigine, levetiracetam, pregabalin, phenobarbital, phenytoin and its metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin, retigabine (ezogabine) and its metabolite N-acetyl retigabine, rufinamide, stiripentol, topiramate, tiagabine, valproic acid, vigabatrin and zonisamide were detected and quantitated in postmortem whole blood, serum and plasma using the transferred method. This method was suitable for routine forensic toxicological analysis and therapeutic drug monitoring. All AEDs were detected and quantified within 17 minutes without endogenous interferences. The correlation coefficient (R^2) was greater than 0.994 for all AEDs with accuracy ranging from 90 to 113% and precision < 14.4% for all analytes. The recovery ranged from 70% to 98%. No carryover was observed in a blank control injected after the highest standard and the matrix effect was acceptable and ranged from 80% to 120%.

6 Application of a Validated Method For the Analysis of AEDs in Biological Matrices

6.1 Introduction

The aim behind any method development and validation is its applicability to authentic case samples and to insure the accuracy and precision of any drug quantification. In order to prove the applicability of the method developed in Chapter 5, the method was verified with an anonymous set of 467 biological samples that had previously been quantified by NMS Labs, Pennsylvania, US using a variety of different analytical techniques (GC/MS, HPLC and LC/MS/MS) and results were compared with the reference lab.

6.2 Methodology

467 previously tested samples which had been collected over a period of 3 months from 4th of December 2013 to 25th of February 2014 were re-tested using the validated method. Biological matrices received were whole blood (7 samples), plasma (7 samples) and serum/plasma (453 samples). Samples were separated depending on matrix type and then classified into groups according to the AED tested by the reference lab (Table 6-1).

Table 6-1: The Number of AED Samples Sent by the Reference Lab For Each Matrix.

Drug	Blood	Plasma	Serum/Plasma	Total No. of Samples
GBP	1	0	72	73
LAC	0	1	49	50
LTG	1	0	43	44
CBZ	0	0	42	42
PGR	0	5	36	41
RFM	0	0	39	39
TPR	0	0	38	38
PBT	0	0	35	35
ZNS	0	0	29	29
LEV	1	0	25	26
PHT	3	0	12	15
TIG	0	0	10	10
RTG	0	1	8	9
VPA	0	0	8	8
STP	1	0	5	6
VIG	0	0	2	2
Total	7	7	453	467

The majority of samples received were labelled serum/plasma indicating that the samples could be quantified using calibrators prepared in either serum or plasma. Only 7 samples

were requested to be specifically tested in plasma. All serum and plasma samples were tested by the reference lab for therapeutic drug monitoring purposes. Only 7 whole blood samples were received as forensic cases. All samples were kept in the fridge at a temperature between 4-8 ºC until the time of analysis.

Calibration curves, QCs and samples were extracted using protein precipitation in methanol as detailed previously (see 5.2.3, 5.2.4 and 5.2.5). A freshly extracted calibration curve in the matched matrix was prepared with each batch of samples with two QCs at a low and a high concentration. When a large number of samples were prepared for analysis in one batch (40-80 samples per batch) QCs were re-injected every 20 samples to ensure that the accuracy and precision of the calibration curve were still within the acceptable ranges.

6.2.1 Method Comparison and Statistical Methods

AED concentrations measured by the reference lab in blood, plasma and serum were compared with AEDs measured by the transferred method. The comparative data was used to evaluate the performance of the transferred LC/MS/MS method. Since the true concentrations were not known, concentrations quantitated by the reference lab were taken as the best estimate available. For each AED, LC/MS/MS concentrations were plotted against their values obtained by the reference lab. Using Minitab® 17 (Minitab LTD, UK), Pearson correlation, an estimated 95% confidence interval and a regression equation describing the line of best fit between the results of two methods was calculated and the standard error of this regression slope determined for each drug.

However, several authors have agreed that the Pearson correlation and the test of significance (95% confidence interval) may be misleading and do not reflect the actual agreement between two methods (282-287). In the case of the Pearson correlation, the results obtained by the two labs could be highly correlated with a systematic difference between them. It has been discussed how a high correlation may be associated with a considerable lack of agreement between two instruments (283, 285). Also, the range of the results significantly affects the value of the correlation coefficient: the higher the range, the higher the value of the correlation coefficient (285, 287). The test of significance is aimed at detecting the difference not the equivalence, hence, it is considered irrelevant to the question of agreement. Furthermore, this test is not applicable in this study due to the small number of samples for some drugs (e.g. vigabatrin: 2 samples, stiripentol: 6 samples) and

due to the many variations involved; two different laboratories, many drugs, a variety of instruments used by the reference lab for AED analysis, different operators and time variation between tests (samples were tested over a 3 month period by the reference lab whereas all samples were analysed over 4 weeks using the transferred method).

Bland and Altman suggested using a plot, with bias and precision statistics, to determine agreement between methods. The Bland-Altman plot considers the proportion between the magnitude of measurements and the error graphically, but not quantitatively. The plot uses the difference between the two methods against their means. This allows investigation of any possible relationship between the measurement error and the true value. Since the true value is not known, the mean of the two measurements is the best estimate available. Consequently, agreement between the two measurements was tested by calculating the systemic error (bias), and the 95% limits of agreement as bias ± 2 SD, as described by Bland and Altman (283).

6.2.2 Instrumentation

Samples provided were initially tested by the reference lab using a variety of analytical instrumentation with different calibration ranges and limits of quantification. A summary of the information provided regarding AED analysis techniques and method LOQs is given in Table 6-2.

Therapeutic concentrations and transferred method LOQs have been included for comparison. Although LOQs for most AEDs of interest were higher using the transferred method, they were still acceptable and below the lowest TDM concentrations. Some drugs such as carbamazepine, levetiracetam, retigabine and its metabolite have more than one LOQ. The LOQ for these depends on the method used by the reference lab. For instance, carbamazepine is tested using HPLC for total CBZ and CBZO and using immunoassay analysis for free CBZ and CBZO. This depends on the clinicians' requirements. The reference lab does not test for oxcarbazepine and eslicarbazepine acetate as parents drugs. Instead, they test for their metabolite 10-hydroxycarbazepine (MHD or LIC) which is a racemic mixture of S and R-licarbazepine (S-LE and R-LE). Phenytoin is also tested as a parent drug or as an active metabolite of fosphenytoin. Fosphenytoin is a phosphate ester pro-drug of PHT, which does not have significant pharmacological activity (288). Therefore, clinical monitoring is concerned only with the plasma concentrations of the derived PHT. p-HPPH is not tested by the reference lab. However, the transferred method

tests for p-HPPH and LIC as well as their parent drugs phenytoin, oxcarbazepine and eslicarbazepine acetate.

Table 6-2: A Summary of AED Analysis Techniques Used by the Reference Lab and Methods LOQs Compared to the Transferred Method.

AEDs	Therapeutic Concentration (mg/L)	Method LOQ	Ref Lab LOQ	Ref Lab Method
CBZ	1.7-15	0.5	2/0.5/0.2	HPLC/IA
CBZO	0.5-2.0	0.5	0.2	HPLC/IA
ESL	10.0-26.0	0.5	N/A	N/A
GBP	5.0-9.0	0.5	0.1	LC/MS/MS
LAC	2.5-14.0	0.5	0.5	HPLC
LEV	10.0-40.0	5	1/2/5	HPLC
LIC/S-LC	6.0-25.0	0.5	N/A	HPLC
LTG	2.3-5.6	1	0.2	HPLC
OXC	0.05-1.2	0.1	N/A	N/A
PBT	10.0-40.0	2.5	0.5	GC/MS
PGR	1.0-5.0	1.0	0.1	LC/MS/MS
PHT	7.0-20.0	1.0	0.5	HPLC
p-HPPH	1.0-40.0	1.0	N/A	N/A
RFM	2-7	0.5	0.5	LC/MS/MS
RTG	0.51-1.85	0.05	0.04/0.4	LC/MS/MS
NA-RTG	0.015-0.2	0.1	0.04/0.4	LC/MS/MS
STP	4-22	0.5	0.1	HPLC
TIG	0.03-1	0.05	0.004	LC/MS/MS
TPR	2.4-27	0.5	0.2	LC/MS/MS
VIG	18-77	5	1	LC/MS/MS
VPA	50-100	5.0	2	HPLC/IA
ZNS	20-28	1.0	1	HPLC

6.3 Results and Discussion

6.3.1 Method Comparison

The method was verified with a set of 467 samples that had previously been quantitated during routine case analysis. Six samples were requested to be tested for 2 AEDs. Another 18 samples were requested to be tested for metabolites as well as parent drugs; 9 samples for carbamazepine epoxide and 9 samples for N acetyl retigabine. Thus, the total number of requested AEDs and their metabolites was 491 tests. In Table 6-3, the confirmed AEDs column represents the number of tests confirmed by the transferred method. Due to the simultaneous analysis of 22 AEDs, the transferred method detected an additional 493

AEDs. Hence, the total number of AEDs detected by the transferred method was 970 (482 confirmed by both methods plus an additional 493).

Out of 482 confirmed AEDs, only 412 were compared with the reference laboratory values. Comparison of the remaining samples was not possible because some of the drugs such as carbamazepine, carbamazepine epoxide and valproic acid were analysed for the free drug (not bound to blood proteins) by the ref lab whereas the transferred method tests for the total concentration (free and protein bound). For other drugs like phenobarbital, the reference laboratory values for 5 samples were not available at the time of the project.

Table 6-3: The Number of Positive AEDs Tested by the Transferred Method in Blood, Plasma and Serum Compared to the Reference Laboratory.

Drug	No. of Requested AEDs	Confirmed AEDs	Paired Results	Additionally Detected AEDs	Total AEDs
CBZ	42	42	10	23	65
CBZO	9	9	5	34	43
GBP	73	73	73	15	88
LAC	51	50	49	11	61
LEV	26	25	25	88	113
LTG	45	43	42	44	87
LIC/SLE	0	0	0	21	21
OXC	0	0	0	19	19
PBT	35	35	30	38	68
PGR	41	38	38	6	44
PHT	15	15	15	57	72
RFM	39	38	38	2	40
RTG	9	9	0	1	10
NA-RTG	9	9	0	1	10
STP	6	6	5	0	6
TIG	10	10	10	0	10
TPR	41	41	41	34	75
VIG	2	2	2	6	8
VPA	8	8	1	67	75
ZNS	30	29	28	26	55
Total	**491**	**482**	**412**	**493**	**970**

There were 21 positive samples for licarbazepine and 19 positive samples for oxcarbazepine but eslicarbazepine acetate analysis gave negative results. As these samples were not tested for these drugs by the reference laboratory, it was not possible to determine whether the compounds found were oxcarbazepine and its metabolite licarbazepine or if

they were eslicarbazepine acetate with its two metabolites oxcarbazepine and S-licarbazepine. Thus, those samples were also excluded from the method comparison statistics.

Retigabine and N-acetyl retigabine were excluded as well due to a stability issue. N-acetyl retigabine concentrations decreased considerably from the time samples were tested by the ref lab to the time tested by the transferred method (around one month) whereas retigabine increased. In order to investigate whether the variation was due to stability of the drugs or an analysis performance issue, the samples were retested by the reference laboratory. The concentrations were found to decrease further for N-acetyl retigabine and increased for retigabine, probably due to the degradation of N-acetyl retigabine to its parent drug. This supports the stability results conducted as part of the validation (see 5.3.9). Retigabine increase was not been detected during the stability study, however, this may be due to the short study period (24 hours) (Table 6-4).

Table 6-4: RTG and NA-RTG Fridge Stability Over a Two Month Period.

Sample No.	RTG Results* (mg/L)			NA-RTG Results* (mg/L)		
	Analysis 1	Analysis 2	Analysis 3	Analysis 1	Analysis 2	Analysis 3
1	8.40	16.92	16.10	4.30	4.79	3.40
2	0.89	1.67	1.10	0.63	0.21	0.15
3	0.87	1.81	2.20	9.10	0.49	0.45
4	0.97	1.25	1.90	9.70	0.37	0.27
5	1.30	2.95	3.20	9.40	0.65	0.44
6	1.20	0.30	0.00	0.64	0.07	0.00
7	2.00	0.70	0.00	1.00	0.56	0.00
8	0.68	0.68	1.70	0.61	0.28	0.27

* Analysis 1 and 3 were conducted by the reference laboratory whereas analysis 2 was conducted using the transferred method.

Paired results (412 samples) were used to create a regression equation describing the line of best fit between two methods. The correlation coefficient for comparison of the quantitation results with the reference laboratory methods was very good, giving an R^2 of 0.961 (Figure 6-1). No interferences were observed with any of the case samples.

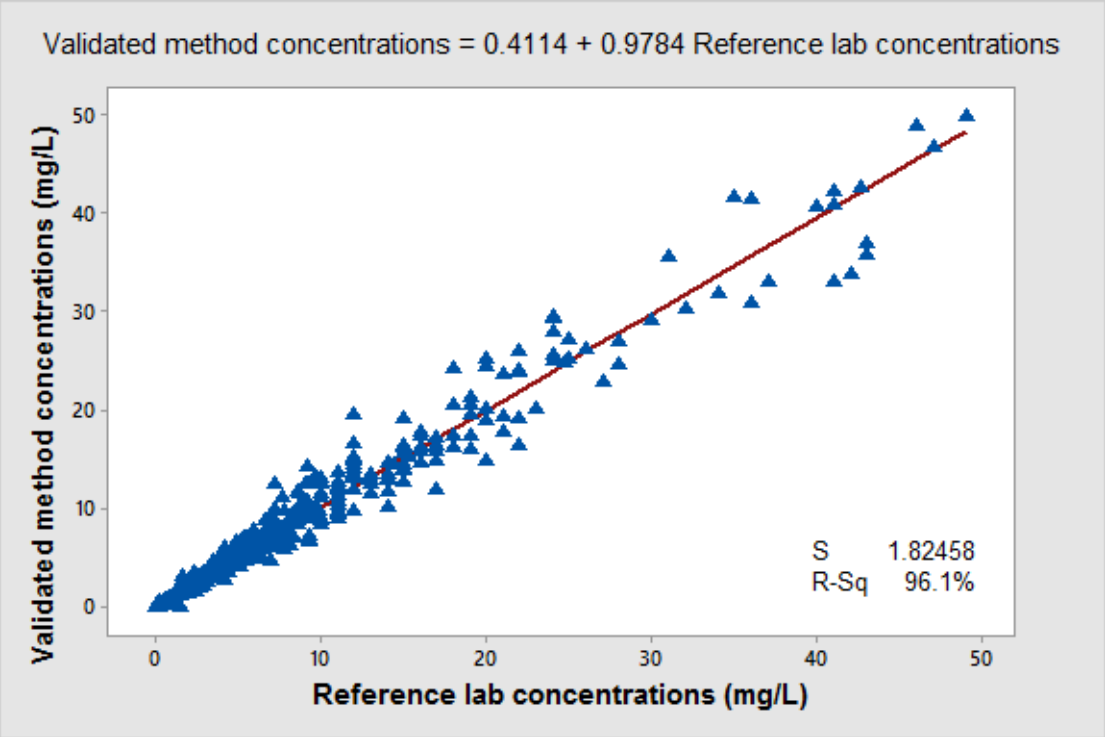


Figure 6-1: Correlation of AED Concentrations Measured with the Transferred Method Versus the Concentrations Measured by the Reference Laboratory for 412 Confirmed AEDs.

Regression equations, Pearson correlations, medians, means and standard deviations were calculated for each drug. A summary of the statistical data is presented in Table 6-5and Table 6-6. Median, Mean and Standard deviation for all AEDs were very similar for both methods. There was no significant difference between the AEDs values measured by the transferred method and the reference laboratory (median: 6.53 mg/L, mean 9.56 ± 9.19 mg/L vs median: 6.65 mg/L, 9.16 ± 9.22 mg/L, respectively, 95% confidence interval, CI: 0.0169, 0.3729).

Table 6-5: Regression Equation and Pearson Correlation for AEDs Tested by the Transferred Method and the Reference Laboratory.

Drug	n	Regression Equation	Pearson Correlation
CBZ	15	Validated method concentration = 0.700 + 1.068 Reference lab concentration	0.980
GBP	72	Validated method concentration = 0.125 + 0.868 Reference lab concentration	0.992
PGR	33	Validated method concentration =-0.076 + 0.933 Reference lab concentration	0.986
LEV	24	Validated method concentration = 2.828 + 0.997 Reference lab concentration	0.982
ZNS	28	Validated method concentration =-0.175 + 1.010 Reference lab concentration	0.989
RFM	35	Validated method concentration = 2.288 + 0.817 Reference lab concentration	0.977
LAC	49	Validated method concentration = 0.932 + 0.939 Reference lab concentration	0.970
LTG	41	Validated method concentration = 0.570 + 0.981 Reference lab concentration	0.988
PBT	28	Validated method concentration =-0.937 + 0.986 Reference lab concentration	0.983
TPR	40	Validated method concentration = 0.027 + 1.210 Reference lab concentration	0.987
PHT	12	Validated method concentration =-0.375 + 1.051 Reference lab concentration	0.979
TIG	9	Validated method concentration =-0.003 + 1.322 Reference lab concentration	0.994

Table 6-6: Median, Mean and Standard Deviation for 18 AEDs Tested by the Transferred Method and the Reference Laboratory.

Drug*	Transferred Method				Reference Lab			
	n	Median (mg/L)	Mean (mg/L)	SD (mg/L)	n	Median (mg/L)	Mean (mg/L)	SD (mg/L)
AEDs	412	6.53	9.56	9.19	412	6.65	9.16	9.22
CBZ	65	7.5	8.7	5.1	10	8.50	7.14	1.48
CBZO	43	2.41	3.9	5.17	5	1.8	2.8	3.6
GBP	88	3.8	4.9	4.6	73	4.00	4.79	3.67
LAC	61	6.7	7.49	4.51	49	6.90	7.21	4.62
LEV	113	28.1	34.1	24.4	25	14.68	17.32	10.05
LIC/S-LE	20	21.71	21.96	9.77	0	n/a	n/a	n/a
LTG	87	6.0	7.1	6.0	42	4.40	5.62	4.41
OXC	19	0.18	0.24	0.40	0	n/a	n/a	n/a
PBT	68	15.0	19.9	16.8	30	17.00	18.44	12.20
PGR	44	2.5	4.2	4.7	38	2.60	4.44	4.49
PHT	72	6.5	7.9	6.4	15	11.75	8.47	4.09
RFM	40	13.5	16.96	10.18	38	14.00	16.08	10.33
STP	6	6.52	8.33	4.19	5	5.15	6.55	3.68
TIG	10	0.03	0.05	0.05	10	0.03	0.04	0.04
TPR	75	6.9	7.4	5.7	41	4.50	5.28	4.08
VIG	8	5.5	7.5	6.4	2	n/a	n/a	n/a
VPA	75	83.1	82.8	32.4	1	n/a	n/a	n/a
ZNS	55	18.4	20.4	14.1	28	16.00	3.18	16.81

*No positive results for eslicarbazepine acetate and p-HPPH. RTG and NA-RTG excluded from the results due to a stability issue.

Even though both methods showed a high correlation for each AED (> 96%); the results do not reflect the agreement between them. Bland and Altman plotting in the current study showed that the mean difference between the transferred method and the reference laboratory was 0.21±1.83 mg/L indicating that the transferred method measured slightly higher concentrations than the reference lab methods and that the scatter increases when the concentration is greater than 10 mg/L. The lower and upper levels of agreement were -3.38 and 3.81. Out of 412 samples, only 27 samples were outliers (~7% of the total number of compared tests). Mean bias (±SD) and limits of agreement for each AED (95% CI) are summarized in Table 6-7. (See Appendix 6-1 for correlation and Bland-Altman Graphs for each drug).

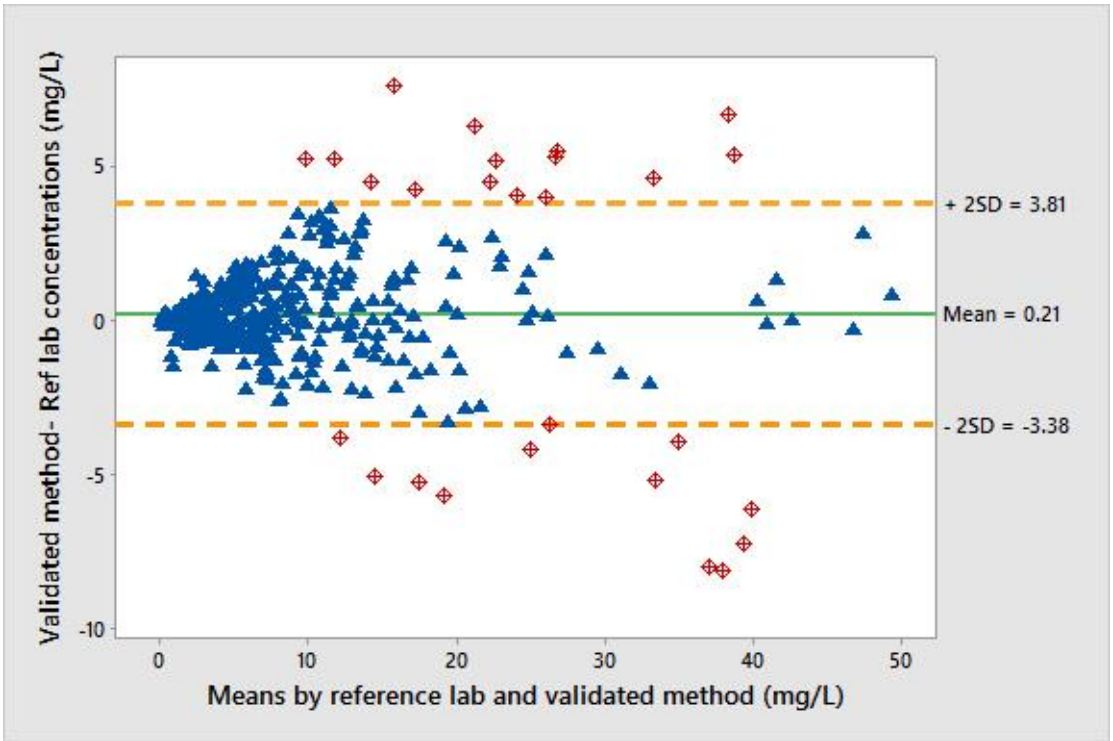


Figure 6-2: Bland-Altman Plot of the Validated Method Concentrations and the Reference Laboratory Concentrations.

Table 6-7: Mean Bias, Standard Deviation and Limits of Agreement at 95% Confidence Interval for AEDs.

Drug	n	Mean Bias ± SD (mg/L)	95% Confidence Interval (Limits of Agreement)
AEDs	412	0.21 ± 1.83	-3.38 - 3.81
CBZ	15	1.18 ± 1.40	-1.57 - 3.94
GBP	72	-0.51 ± 0.64	-1.76 - 0.74
PGR	33	0.37 ± 0.77	-1.88 - 1.13
LEV	24	2.76 ± 2.28	-1.61 - 7.32
ZNS	28	0.04 ± 2.56	-4.98 - 5.05
RFM	35	0.51 ± 2.73	-5.86 - 4.85
LAC	49	0.50 ± 1.12	-1.7 - 2.69
LTG	41	0.35 ± 0.68	-0.99 - 1.69
PBT	28	-1.20 ± 2.27	-5.65 - 3.25
TPR	40	1.13 ± 1.53	-1.15 - 3.42
PHT	12	0.05 ± 0.91	-1.73 - 1.84
TIG	9	0.00 ± 0.03	-0.06 - 0.06

6.3.2 AED Prevalence and Concentration Range

The number of AEDs detected with concentrations below, within and above the therapeutic ranges is detailed in Table 6-8. Interestingly, 216 samples were detected with AED

concentrations higher than the therapeutic ranges (22.3% of the total 970 detected). S-licarbazepine, levetiracetam, rufinamide, carbamazepine epoxide, and valproic acid accounted for most of these in 45, 34, 32, 27 and 20 samples respectively. Considering that only 9 samples were requested to be tested for carbamazepine epoxide, this means that more than 20 samples have non controlled high concentrations. Similarly, S-licarbazepine concentrations were high in 45 samples and none of these were requested for therapeutic drug monitoring. On the other hand 196 samples were below the therapeutic concentrations (20.2%). Phenytoin, zonisamide, gabapentin and phenobarbital account for most of these with 39, 31, 27 and 21 samples respectively. Overall more than 42% of samples were not within the published therapeutic ranges and were not requested to be tested.

Table 6-8: AED Concentration Ranges, Number of AEDs Below, Within and Above the Therapeutic Range.

Drug	Therapeutic Concentration (mg/L)	Sample Concentration Range (mg/L)	No. of Samples **below** Therapeutic range	No. of Samples **Within** Therapeutic range	No. of Samples **above** Therapeutic range
CBZ	1.7-15	0.4-20.16	1	60	4
CBZO	0.5-2.0	0.04-23.86	0	16	27
GBP	5.0-9.0	0.2-24.58	27	53	8
LAC	2.5-14.0	0.5-24.98	8	48	5
LEV	10.0-40.0	0.97-122.11	7	72	34
LIC/S-LE	6.0-25.0	3.16-39.71	17	25	45
LTG	2.3-5.6	0.5-44.2	0	16	5
OXC	0.05-1.2	0.02-1.95	0	18	1
PBT	10.0-40.0	2.68-107.11	21	41	6
PGR	1.0-5.0	0.3-18.79	4	28	12
PHT	7.0-20.0	0.38-32.62	39	32	1
RFM	2-7	2.79-40.8	0	8	32
RTG	0.51-1.85	0.3-16.92	0	9	1
NA-RTG	0.015-0.2	0.21-4.79	1	6	3
STP	4-22	4.0-14.8	0	6	0
TIG	0.03-1	0.006-0.131	5	5	0
TPR	2.4-27	0.44-32.10	19	55	1
VIG	18-77	1.07-19.64	7	1	0
VPA	50-100	13.23-200.7	9	46	20
ZNS	20-28	2.0-76.12	31	13	11
Total number of samples			196	558	216

Out of the 467 samples, only 6 samples were requested to be tested for more than one drug. However, sample reanalysis revealed that 43% of these samples had only one AED, whereas 57% of the samples had at least 2 AEDs (Figure 6-3). Half of the samples had either 2 AEDs (30%) or 3 AEDs (20%). Interestingly, 33 serum samples had 4, 5 and 6 AEDs in 5%, 2% and 0.2% of the cases respectively. Plasma samples had a maximum of 3 AEDs per sample whereas blood samples had only one AED except for one sample which had 2 AEDs (Table 6-9).

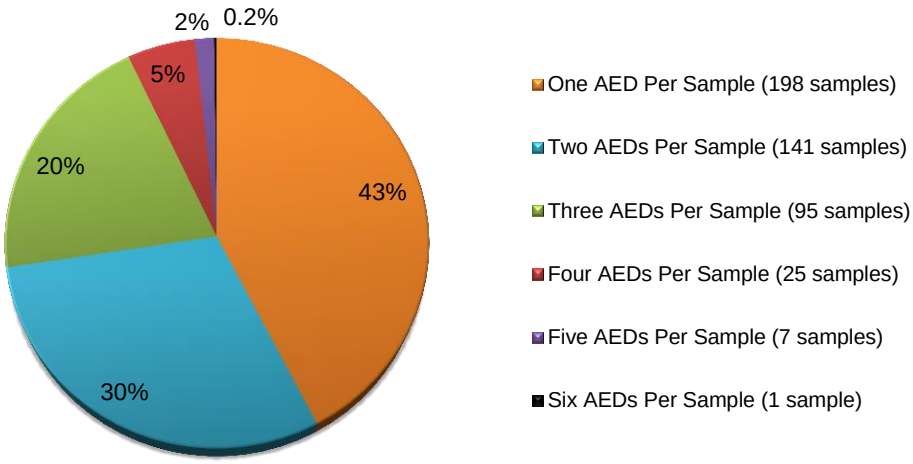


Figure 6-3: Percentage of AEDs Detected Per Sample.

Table 6-9: Number of AEDs Detected Per Sample for Each Matrix.

AEDs No.	Blood	Plasma	Serum
1	6	3	189
2	1	1	139
3	0	3	92
>4	0	0	33
Total	7	7	453

AEDs detected in samples with 5-6 AEDs are presented in Table 6-10. Lamotrigine, levetiracetam and phenytoin dominated in 6, 6 and 7 of the 8 samples respectively, followed by phenobarbital (4 samples), lacosamide, valproic acid and zonisamide (3 samples each), vigabatrin, topiramate and rufinamide (2 samples each), gabapentin, pregabalin, carbamazepine and its metabolite carbamazepine epoxide (1 sample each).

The concentrations of most AEDs in these 8 samples are within the therapeutic ranges except for lamotrigine in sample 1 and 5; its concentration was higher than its therapeutic range (> 5.6 mg/L) with concentrations of 13.95 and 8.28 mg/L respectively. Phenytoin concentrations were significantly lower than its therapeutic level (< 7 mg/L) in 5 out of 7

samples. The zonisamide concentration was lower than its therapeutic level in sample 3 (7.88 mg/L). However, all of these samples were requested to be tested for only 1 AED by clinicians except for sample 8 which was requested to be tested for 2 AEDs.

Table 6-10: AED Concentrations in 8 Serum Samples with 5-6 Positive AEDs.

Sample No.	1	2	3	4	5	6	7	8
AED No.	6	5	5	5	5	5	5	5
AED 1	LTG	LTG	LTG	LTG	LTG	LTG*	PHT*	PBT
(mg/L)	13.95	5.64	6.69	5.15	8.28	5.17	9.78	4.12
AED 2	PHT	PHT	PHT	PHT	PHT	PHT	PBT	LEV
(mg/L)	2.60	12.43	1.77	2.28	1.04	0.87	26.66	12.67
AED 3	PBT	PGR*	RFM*	LEV	LEV	PBT	LEV	VPA*
(mg/L)	6.00	0.70	10.29	31.06	42.96	19.43	37.52	86.95
AED 4	LEV	TPR	VPA	RFM*	TPR	LEV	VPA	VIG*
(mg/L)	66.70	6.04	67.49	16.30	7.38	30.02	58.67	15.30
AED 5	LAC*	ZNS	ZNS	LAC	CBZ*	LAC	GBP	ZNS
(mg/L)	8.84	35.61	7.88	6.44	9.17	7.62	4.55	42.85
AED 6	VIG							
(mg/L)	19.64							
Metabolites					CBZO*			
(mg/L)					2.41			

*Shaded cells refer to the AED requested to be tested by clinicians.

Similarly, Table 6-11 shows 25 serum samples with 4 positive AEDs per sample (139 AED tests). The AED 1 column represents the concentrations of AEDs that were requested by clinicians. Once again, all these samples were requested to be tested for only one drug except sample number 14 which was tested for 2 AEDs; levetiracetam and valproic acid.

Out of 25 samples, only 5 samples have AED therapeutic concentrations whereas 20 samples have at least one AED below or above the recommended therapeutic range. Out of 139 tests, 33 tests were not within recommended concentrations (23.7%). Although TDM guidance does not require blood monitoring for new AED generations, TDM for old generations such as carbamazepine, phenytoin, phenobarbital, lamotrigine and valproic acid is still a requirement due to their adverse effects (See 1.8, Chapter 1). This table shows some old AEDs with concentrations below or higher than their levels required for epilepsy maintenance but their analysis was not requested for any of these samples. Samples 29 and 32 were positive for S-licarbazepine but as discussed earlier it was not

possible to decide whether this was resulting from oxcarbazepine or eslicarbazepine acetate treatment.

Table 6-11: AEDs Concentrations in 25 Serum Samples with 4 Positive AEDs.

Sample No.	AED 1 (mg/L)*		AED 2 (mg/L)		AED 3 (mg/L)		AED 4 (mg/L)		Metabolite (mg/L)	
9	LAC	7.12	ZNS	16.19	TPR	1.57	PHT	16.34		
10	LAC	4.70	LEV	28.48	PHT	4.15	VPA	35.59		
11	LAC	7.78	ZNS	15.12	PBT	41.67	PHT	4.79		
12	LAC	5.27	TPR	0.51	PHT	6.49	VPA	53.37		
13	LAC	2.77	LTG	5.40	TPR	0.99	CBZ	6.45	CBZO	6.10
14**	LEV	24.80	LTG	3.10	PBT	6.10	VPA	67.70		
15	LEV	24.49	LTG	0.52	VIG	1.45	PHT	6.86		
16	LTG	14.13	LEV	3.66	PHT	1.05	VPA	87.18		
17	RFM	33.88	LEV	46.36	TPR	10.35	OXC	0.17	SLE	24.46
18	RFM	30.80	LEV	29.70	LTG	14.96	VPA	97.91		
19	RFM	5.10	LEV	28.26	LTG	7.68	TPR	8.01		
20	RFM	30.25	LEV	65.55	TPR	10.36	VPA	129.34		
21	RFM	7.04	LEV	22.93	LTG	12.78	VPA	57.03		
22	PBT	5.51	LEV	12.40	TPR	2.66	CBZ	18.59	CBZO	9.74
23	PBT	0.60	LEV	22.26	LTG	1.83	CBZ	12.74	CBZO	5.05
24	PGR	3.04	LTG	8.10	LAC	8.88	PHT	2.13		
25	PGR	7.82	ZNS	17.42	LAC	6.08	PHT	19.12		
26	PGR	1.66	LEV	15.70	ZNS	19.28	PBT	18.68		
27	RTG	1.67	LTG	9.99	LAC	9.94	PBT	5.17	NRTG	0.21
28	STP	6.40	ZNS	21.9	RFM	40.80	PBT	24.9		
29	TIG	0.006	LEV	46.88	PBT	9.37	OXC	0.00	SLE	13.88
30	TIG	0.055	LTG	11.83	GBP	24.58	TPR	11.03		
31	TPR	9.80	VIG	<5(1)	GBP	4.70	LTG	1.30		
32	TPR	3.30	GBP	2.50	VPA	133.4	OXC	0.21	SLE	14.8
33	VPA	91.40	LEV	33.7	PBT	8.00	VPA	91.4		

* AED1 column refers to AEDs requested to be tested.

**Sample 14 was requested to be tested for 2 AEDs, LEV and VPA.

- Shaded Cells refer to samples with concentrations below or above the therapeutic range.

Table 6-12 summarizes AEDs concentrations in plasma and postmortem blood. AEDs in plasma samples were all within the therapeutic ranges. Post mortem blood cases were requested to be tested for one AED and were confirmed with the transferred method except

for sample 1 which had 2 AEDs (STP: 1.90 mg/L and VPA: 28.43 mg/L). However, VPA is within therapeutic range. More details on AED concentrations in serum samples with 3, 2 and 1 AEDs are presented in Table 6-13, Table 6-14 and Table 6-15.

Table 6-12: AEDs Concentrations in 7 Postmortem Blood and 7 Plasma Samples.

Matrix	Sample No.	No. of AED	AED 1 (mg/L)*		AED 2 (mg/L)		AED 3 (mg/L)		Metabolite (mg/L)	
Plasma	1	3	RTG	4.2	GBP	8.51	PBT	14.13	NRTG	0.6
Plasma	2	3	PGR	1.5	LEV	18.5	TPR	5.42		
Plasma	3	3	PGR	6.7	ZNS	41.7	LTG	9.44		
Plasma	4	2	LAC	8.56	LEV	73.1				
Plasma	5	1	PGR	2.1					SLE	7.9
Plasma	6	1	PGR	1.9						
Plasma	7	1	PGR	5.1						
Postmortem blood	1	2	STP	1.9	VPA	28.4				
Postmortem blood	2	1	GBP	8.4						
Postmortem blood	3	1	LEV	17.5						
Postmortem blood	4	1	LTG	6.2						
Postmortem blood	5	1	PHT	3.3						
Postmortem blood	6	1	PHT	13.2						
Postmortem blood	7	1	PHT	15.2						

* AED1 column refers to AEDs requested to be tested.
- Shaded Cells refer to samples with concentrations below or above the therapeutic range.

As no case history was available, it was difficult to interpret the variation in concentrations or investigate the reason behind the high number of AEDs in some samples. It may be as a result of emergency treatment (hospital samples) or due to a change in the treatment regime which requires decreasing the concentration of the old drugs and increasing the concentration of the new drugs gradually in order to avoid any seizure triggers or any withdrawn symptoms that some drugs might have. Alternatively, it might be due to patients abusing these drugs or being medicated for something other than epilepsy with

different therapeutic level requirements such as neuropathic pain, migraine, bipolar disorder and other psychiatric disease (28).

In general, clinical and forensic laboratories analyse AEDs only when requested by clinicians or pathologists and usually the drugs are specified, although 20% of epileptic patients are using a polytherapy of AEDs (149). For example, 22 cases have been tested for only one drug, although the sample had another three drugs detected by the validated method. The analysis cost and time in addition to the ability to afford state of art techniques can be obstacles, which may affect the number of tests requested. Therefore, the development of the simultaneous analysis of AEDs using a small sample volume and a simple extraction procedure may improve the TDM of these drugs and can play an important role in enhancing the quality of life for epileptic people. With increased awareness of poly-AED use, the incidence of SUDEP could be reduced. As mentioned previously, different risk factors account for this phenomenon such as the seizures frequency, the number of AEDs taken concomitantly (65, 66) and the variability of AED ingestion over time (67). Finally, by monitoring all the medications taken by patients and observing any changes in concentrations, which may result from drug interactions, pharmacogenetic variations or from other AEDs being taken concomitantly by patient either with or without prescription, greater clinical care can be given to these patients.

6.4 Conclusion

A simple, accurate and cost-effective LC/MS/MS method was transferred for the simultaneous quantification of 15 AEDs and 2 metabolites. The method was extended to include another 5 compounds, 3 AEDs and 2 metabolites, so the total number of detected and quantitated AEDs by the transferred method was 22 including metabolites. The method was validated in postmortem blood, serum and plasma which made it suitable for both routine forensic toxicology and TDM. It was successfully verified using 467 authentic case samples with a correlation higher than 96%. Bland-Altman plots showed good agreement between both methods with a mean difference of 0.21 ± 1.83 mg/L and limits of agreement were -3.38 - 3.81, 95% CI.

The AED analysis successfully quantified 1-6 AEDs per sample although most of these samples were requested to be tested for only 1 AED. Simultaneous analysis presents an important tool for TDM where AEDs are taken concomitantly and concentration variations could significantly affect the quality of life for people with epilepsy related conditions.

Table 6-13: AED Concentrations in 92 Serum Samples with 3 Positive AEDs.

Sample No.	AED 1 (mg/L)*		AED 2 (mg/L)		AED 3 (mg/L)		Metabolite (mg/L)	
34	CBZ	5.66	GBP	3.04	PBT	2.81	CBZO	1.15
35	CBZ	10.57	LEV	32.96	VPA	101.03	CBZO	23.86
36	CBZ	10.58	TPR	7.70	VPA	94.14	CBZO	17.26
37	CBZ	13.60	LEV	37.68	VPA	72.50	CBZO	23.19
38	CBZ	5.03	LEV	1.59	VPA	73.45	CBZO	2.93
39**	GBP	1.63	LEV	33.19	LTG	12.43		
40**	GBP	3.00	PBT	10.45	TPR	2.48		
41	GBP	12.96	VIG	1.86	CBZ	13.87	CBZO	3.37
42	GBP	2.54	PHT	4.52	VPA	22.08		
43	GBP	3.02	PHT	7.23	CBZ	6.47	CBZO	2.08
44	GBP	6.27	LEV	15.92	VPA	91.00		
45	GBP	7.21	LTG	1.22	RTG	0	NRTG	0.37
46	LAC	5.76	PHT	12.77	VPA	58.32		
47	LAC	4.26	PBT	16.75	OXC	0.22	SLE	16.66
48	LAC	10.39	LEV	28.12	VPA	78.49		
49	LAC	5.80	LEV	24.45	PHT	6.99		
50	LAC	4.71	ZNS	13.44	LTG	4.29		
51	LAC	7.99	LEV	74.52	VPA	177.56		
52	LAC	6.76	ZNS	16.04	LTG	2.65		
53	LAC	7.46	LEV	40.25	LTG	12.30		
54	LAC	6.65	PHT	5.69	VPA	46.17		
55	LAC	6.76	PHT	0.68	OXC	1.95	SLE	20.49
56	LAC	2.59	LTG	7.56	TPR	6.15		
57	LAC	6.92	LTG	3.78	PBT	6.57		
58	LAC	4.79	PHT	9.62	VPA	101.51		
59	LAC	3.22	LEV	17.71	PHT	18.67		
60	LAC	6.83	LEV	22.49	VPA	109.91		
61	LAC	6.53	LTG	8.12	CBZ	9.68	CBZO	3.45
62	LAC	7.07	TPR	9.14	VPA	58.63		
63	LAC	6.60	LEV	11.48	PHT	17.17		
64	LAC	5.94	LEV	9.35	PHT	9.93		
65	LAC	3.19	PHT	3.84	CBZ	11.18	CBZO	3.87
66	LAC	13.61	LTG	9.57	TPR	15.03		
67	LEV	9.1	LAC	0.9	PHT	3.2		
68	LEV	41.70	LAC	2.16	PHT	5.00		
69	LTG	11.88	PBT	15.16	VPA	107.49		
70	LTG	4.94	PGR	1.17	LEV	50.32		
71	LTG	6.96	TPR	6.45	VPA	40.55		
72	PBT	6.73	LTG	2.42	TPR	2.97		
73	PBT	42.29	PHT	7.05	VPA	13.23		
74	PBT	49.81	OXC	0.19	VPA	122.80	SLE	15.47
75	PBT	33.04	GBP	21.51	PHT	18.91		
76	PBT	37.11	PHT	7.11	VPA	86.54		
77	PBT	14.52	PGR	1.10	PHT	4.68		
78	PBT	9.83	GBP	2.84	TPR	4.61		
79	PGR	<0.5(0.29)	LEV	40.44	LTG	44.20		
80	PGR	17.44	LEV	95.56	ZNS	25.44		
81	PGR	<0.5(0.46)	ZNS	17.83	PHT	14.55		
82	PGR	18.79	LEV	70.60	ZNS	15.43		

* AED1 column refers to AEDs requested to be tested.

**Samples 39 and 40 were requested to be tested for 2 AEDs, GBP&LTG and GBP &TPR respectively.

- Shaded Cells refer to samples with concentrations below or above the therapeutic range.

Table 6-13: AED Concentrations in 92 Serum Samples with 3 Positive AEDs (continued…).

Sample No.	AED 1 (mg/L)*		AED 2 (mg/L)		AED 3 (mg/L)		Metabolite (mg/L)	
83	PGR	3.70	TPR	10.01	VPA	31.12		
84	PGR	6.13	VIG	1.07	LAC	10.00		
85	PGR	5.91	LEV	22.78	TPR	32.10		
86	PGR	4.15	LEV	63.21	PHT	1.67		
87	PGR	1.20	VIG	5.50	LEV	49.31		
88	PGR	2.51	GBP	0.79	LEV	25.55		
89	PGR	1.92	LEV	48.11	ZNS	23.25		
90	PGR	12.53	LEV	78.85	ZNS	24.47		
91	PGR	8.88	TPR	16.50	VPA	46.46		
92	PGR	4.62	LTG	8.19	TPR	2.08		
93	PGR	3.53	LEV	79.47	LTG	22.13		
94	PGR	1.15	VPA	90.25	CBZ	14.11	CBZO	18.63
95	RFM	5.96	ZNS	29.85	OXC	0.16	SLE	24.23
96	RFM	5.33	LEV	21.37	VPA	71.43		
97	RFM	12.32	GBP	4.47	LTG	7.62		
98	RFM	15.62	PBT	10.73	TPR	6.01		
99	RFM	13.48	TPR	10.54	VPA	89.27		
100	RFM	13.53	LEV	50.07	RTG	1.92	NRTG	0.21
101	RFM	25.58	PBT	39.12	VPA	113.48		
102	RFM	33.00	LEV	16.34	VPA	75.61		
103	RFM	19.39	TPR	10.13	VPA	91.61		
104	RFM	13.23	LTG	10.77	VPA	94.04		
105	RFM	11.09	LEV	33.55	ZNS	25.55		
106	RFM	13.35	LEV	23.75	OXC	0.27	SLE	39.71
107	RFM	5.89	LEV	11.11	PHT	9.98		
108	RFM	20.47	TPR	4.43	VPA	83.14		
109	RTG	16.92	LEV	49.16	PHT	17.58	NRTG	4.79
110	RTG	1.81	LEV	23.66	LTG	6.39	NRTG	0.49
111	RTG	0.30	LEV	104.58	ZNS	12.06	NRTG	0.07
112	RTG	0.70	LTG	6.32	OXC	0.00	SLE	21.71
							NRTG	0.56
113	RTG	0.68	LEV	30.52	LAC	2.34	NRTG	0.28
114	STP	4.0	ZNS	2.0	VPA	106.9		
115	TIG	0.010	TPR	2.21	VPA	62.67		
116	TIG	0.031	VPA	86.47	CBZ	11.63	CBZO	5.90
117	TIG	0.067	LEV	0.97	VPA	83.17		
118	TIG	0.107	LEV	24.47	CBZ	1.92	CBZO	0.00
119	TPR	12.4	OXC	0.24	VPA	113.0	SLE	22.5
120	TPR	14.9	PBT	7.5	VPA	94.7		
121	TPR	1.3	LEV	19.3	PHT	12.2		
122	TPR	11.02	OXC	0.25	VPA	105.67	SLE	22.39
123	TPR	1.16	LEV	10.12	PHT	4.88		
124	VIG	7	PBT	6.9	CBZO	6.0	CBZO	3.1
125	ZNS	11.26	PBT	28.05	VPA	76.17		

* AED1 column refers to AEDs requested to be tested.

**Samples 39 and 40 were requested to be tested for 2 AEDs, GBP&LTG and GBP &TPR respectively.

- Shaded Cells refer to samples with concentrations below or above the therapeutic range.

Table 6-14: AED Concentrations in 139 Serum Samples with 2 Positive AEDs.

Sample No.	AED 1 (mg/L)*		AED 2 (mg/L)		Metabolite (mg/L)	
126	CBZ	12.09	PBT	4.36	CBZO	2.49
127	CBZ	9.12	TPR	1.65	CBZO	5.64
128	CBZ	4.07	PHT	9.40	CBZO	0.96
129	CBZ	6.45	PBT	3.19	CBZO	0.39
130	CBZ	9.83	LEV	14.22	CBZO	3.52
136	GBP	3.86	CBZ	18.36	CBZO	7.54
137	GBP	3.11	CBZ	6.93	CBZO	1.39
139	GBP	4.70	CBZ	5.10	CBZO	0.30
144	GBP	5.40	CBZ	4.74	CBZO	1.17
131	GBP	0.48	PHT	1.99		
132	GBP	2.20	VPA	51.31		
133	GBP	2.15	VPA	75.16		
134	GBP	4.67	PHT	7.91		
135	GBP	8.86	PHT	7.53		
138	GBP	1.66	TPR	7.25		
140	GBP	6.24	CBZ	4.60		
141	GBP	4.84	LTG	1.73		
142	GBP	1.93	PBT	14.42		
143	GBP	6.64	LEV	4.43		
148	LAC	0.51	OXC	0.00	SLE	16.76
152	LAC	8.93	OXC	0.31	SLE	34.81
160	LAC	5.45	OXC	0.04	SLE	6.85
145	LAC	13.83	ZNS	34.26		
146	LAC	8.97	LEV	53.23		
147	LAC	6.16	LEV	21.96		
149	LAC	6.60	LEV	32.09		
150	LAC	7.70	LTG	6.51		
151	LAC	4.85	VPA	101.56		
153	LAC	16.13	LEV	29.49		
154	LAC	1.68	PHT	11.94		
155	LAC	13.42	ZNS	23.97		
156	LAC	11.37	ZNS	8.59		
157	LAC	17.69	LEV	122.11		
158	LAC	16.26	LEV	25.33		
159	LAC	3.56	VPA	60.55		
161	LAC	6.49	VPA	29.22		
162	LAC	5.57	TPR	9.49		
163	LAC	24.98	LEV	53.60		
164	LAC	8.46	LTG	8.62		
165	LAC	11.17	LEV	65.49		
166	LEV	12.50	CBZ	10.90	CBZO	3.75
169	LEV	29.50	CBZ	12.60	CBZO	7.11
170	LEV	120.42	CBZ	4.91	CBZO	3.02
167	LEV	15.25	PHT	32.62		
168	LEV	0.09	PBT	9.92		
171	LEV	17.71	PBT	26.30		
172	LEV	10.56	PBT	19.98		
173	LTG	11.79	VPA	97.95		
174	LTG	0.97	VPA	44.69		
175	LTG	11.89	VPA	80.02		

* AED1 column refers to AEDs requested to be tested.
**Samples 183 and189 were requested to be tested for 2 AEDs.
- Shaded cells refer to samples with concentrations below or above the therapeutic range.

Table 6-14: AED Concentrations in 139 Serum Samples with 2 Positive AEDs (Continued…).

Sample No.	AED 1 (mg/L)*		AED 2 (mg/L)		Metabolite (mg/L)	
176	LTG	14.69	LEV	77.32		
177	LTG	10.03	LEV	50.35		
178	LTG	7.72	LEV	23.02		
179	LTG	6.01	CBZ	6.51	CBZO	1.60
187	LTG	2.91	CBZ	7.66	CBZO	2.06
180	LTG	3.82	PHT	1.81		
181	LTG	5.02	PHT	1.03		
182	LTG	16.45	GBP	12.02		
183**	LTG	8.92	TPR	11.84		
184	LTG	5.89	TPR	>0.5		
185	LTG	1.29	VPA	56.28		
186	LTG	1.77	GBP	1.57		
188	LTG	15.71	VPA	123.82		
189**	LTG	1.88	ZNS	18.92		
190	LTG	1.95	PHT	0.95		
191	PBT	31.91	PHT	15.41		
192	PBT	24.61	PHT	5.02		
193	PBT	11.92	PHT	6.34		
194	PBT	19.15	PHT	1.08		
195	PBT	29.09	PHT	18.71		
196	PBT	23.69	PHT	5.26		
197	PBT	9.78	GBP	3.68		
198	PBT	33.70	LEV	60.09		
199	PBT	46.31	VPA	116.45		
200	PBT	3.45	LEV	34.21		
201	PBT	16.23	PHT	7.85		
202	PBT	11.78	PHT	5.90		
203	PGR	1.14	TPR	9.73		
204	PGR	1.64	LAC	15.72		
205	PGR	4.40	LEV	20.10		
206	PGR	8.29	ZNS	30.60		
207	PGR	1.38	VPA	58.74		
208	PGR	2.46	LTG	7.84		
209	PGR	14.59	GBP	20.11		
210	PGR	0.00	PHT	1.00		
211	PGR	1.52	TPR	9.70		
212	PGR	4.82	ZNS	0.26		
213	PGR	3.46	PHT	16.15		
214	PGR	0.00	TPR	9.86		
215	PGR	2.85	LTG	2.87		
216	PHT	1.42	LEV	12.90		
217	PHT	6.28	LEV	14.25		
218	RFM	11.68	LTG	10.18		
219	RFM	8.03	VPA	99.80		
220	RFM	20.20	LEV	62.04		
221	RFM	12.12	OXC	0.18	SLE	38.08
226	RFM	38.25	OXC	0.13	SLE	21.00
222	RFM	26.14	LTG	1.67		
223	RFM	15.98	PBT	13.98		
224	RFM	26.93	VPA	76.58		

* AED1 column refers to AEDs requested to be tested.

**Samples 183 and189 were requested to be tested for 2 AEDs.

- Shaded cells refer to samples with concentrations below or above the therapeutic range.

Table 6-14: AED Concentrations in 139 Serum Samples with 2 Positive AEDs (Continued…).

Sample No.	AED 1 (mg/L)*		AED 2 (mg/L)		Metabolite (mg/L)	
225	RFM	5.92	LEV	18.45		
227	RFM	9.59	LTG	3.16		
228	RFM	14.12	LEV	30.95		
229	RTG	1.25	VPA	51.76	NRTG	0.37
230	RFM	9.35	PBT	18.24		
231	RFM	35.62	TPR	19.18		
232	RFM	7.48	LTG	14.01		
233	RTG	2.95	PGR	1.56	NRTG	0.65
234	STP	9.9	VPA	118.2		
235	STP	14.8	VPA	200.7		
236	STP	6.5	VPA	120.9		
237	TIG	0.015	CBZ	16.86	CBZO	4.80
238	TIG	0.000	PHT	15.76		
239	TPR	7.4	LAC	0.8		
240	TPR	9.05	VPA	83.07		
241	TPR	9.29	LEV	87.28		
242	TPR	3.10	PHT	0.55		
243	TPR	14.84	PHT	0.42		
244	TPR	8.83	LEV	64.20		
245	TPR	6.10	PBT	20.17		
246	TPR	2.25	LEV	15.44		
247	TPR	12.43	LEV	43.04		
248	TPR	16.36	PBT	39.77		
249	TPR	5.12	LTG	4.19		
250	TPR	16.49	LEV	58.46		
251	TPR	9.43	LEV	63.64		
252	TPR	5.61	LTG	3.08		
253	TPR	1.5	CBZ	7.1	CBZO	1.2
254	TPR	10.0	LEV	15.1		
255	TPR	19.6	OXC	0.41		
256	TPR	4.4	PBT	4.9		
257	VPA	123.90	LEV	32.23		
258	VPA	65.46	TPR	2.84		
259	ZNS	17.3	OXC	0.22	SLE	34.3
260	ZNS	40.6	LEV	10.0		
261	ZNS	40.8	LTG	11.2		
262	ZNS	19.45	LEV	31.59		
263	ZNS	8.81	PHT	18.51		
264	ZNS	15.76	LEV	27.94		

* AED1 column refers to AEDs requested to be tested.

**Samples 183 and189 were requested to be tested for 2 AEDs.

- Shaded cells refer to samples with concentrations below or above the therapeutic range.

Table 6-15: AED Concentrations in 189 Serum Samples with One Positive AED.

Sample No.	AED 1 (mg/L)*		Metabolite (mg/L)		Sample No.	AED 1 (mg/L)*	
265	CBZ	9.60	CBZO	0.66	314	GBP	0.23
266	CBZ	5.61	CBZO	0.23	315	GBP	2.62
267	CBZ	20.16	CBZO	14.32	316	GBP	1.00
268	CBZ	0.40	CBZO	0.00	317	GBP	2.03
269	CBZ	4.66	CBZO	0.36	318	GBP	1.31
270	CBZ	8.14	CBZO	0.59	319	GBP	3.48
271	CBZ	4.40	CBZO	0.54	320	GBP	2.51
272	CBZ	13.48	CBZO	2.90	321	GBP	9.30
273	CBZ	8.27	CBZO	1.47	322	GBP	11.49
274	CBZ	3.28	CBZO	0.09	323	GBP	0.67
275	CBZ	6.23	CBZO	0.16	324	GBP	5.84
276	CBZ	5.81	CBZO	0.07	325	GBP	0.81
277	CBZ	4.19	CBZO	0.34	326	GBP	8.81
278	CBZ	4.91	CBZO	0.04	327	GBP	3.34
279	CBZ	11.07	CBZO	6.06	328	GBP	0.41
280	CBZ	7.02	CBZO	2.00	329	GBP	4.71
281	CBZ	12.49	CBZO	2.29	330	GBP	6.06
282	CBZ	6.02	CBZO	0.72	331	GBP	6.38
283	CBZ	6.37	CBZO	0.81	332	GBP	4.52
284	CBZ	7.48	CBZO	2.21	333	GBP	5.13
285	CBZ	11.21	CBZO	3.20	334	GBP	6.07
286	CBZ	12.66	CBZO	5.12	335	GBP	2.10
287	CBZ	3.83	CBZO	1.18	336	GBP	11.74
288	CBZ	3.16	CBZO	0.32	337	GBP	5.31
289	CBZ	4.34	CBZO	0.40	338	GBP	4.26
290	CBZ	13.56	CBZO	4.39	339	GBP	2.75
291	CBZ	4.27	CBZO	1.01	340	GBP	5.28
292	CBZ	8.98	CBZO	2.44	341	GBP	1.81
293	CBZ	14.71	CBZO	3.61	342	GBP	1.45
294	CBZ	6.91	CBZO	3.03	343	GBP	3.14
295	CBZ	14.39	CBZO	5.62	344	GBP	5.74
296	GBP	2.12			345	GBP	1.93
297	GBP	1.58			346	GBP	6.96
298	GBP	5.82			347	LAC	8.69
299	GBP	17.39			348	LEV	12.8
300	GBP	5.56			349	LEV	14.4
301	GBP	4.00			350	LEV	42.7
302	GBP	1.69			351	LEV	25.18
303	GBP	1.32			352	LEV	24.30
304	GBP	5.28			353	LEV	19.22
305	GBP	2.01			354	LEV	11.47
306	GBP	4.34			355	LEV	13.02
307	GBP	0.80			356	LEV	27.98
308	GBP	9.75			357	LEV	29.30
309	GBP	3.93			358	LEV	11.47
310	GBP	1.43			359	LEV	12.68
311	GBP	3.79			360	LEV	12.75
312	GBP	1.93			361	LEV	41.40
313	GBP	2.82			362	LTG	8.81

* AED1 column refers to AEDs requested to be tested.

- Shaded cells refer to samples with concentrations below or above the therapeutic range.

Table 6-15: AED Concentrations in 189 Serum Samples with One Positive AED (Continued..).

Sample No.	AED 1 (mg/L)*		Sample No.	AED 1 (mg/L)*	
363	LTG	1.37	412	TIG	0.000
364	LTG	4.35	413	TIG	0.131
365	LTG	6.95	414	TPR	12.7
366	LTG	2.13	415	TPR	2.3
367	LTG	7.64	416	TPR	0.8
368	LTG	3.09	417	TPR	1.6
369	LTG	3.33	418	TPR	1.01
370	LTG	5.15	419	TPR	0.44
371	LTG	3.20	420	TPR	5.48
372	LTG	5.48	421	TPR	3.40
373	LTG	6.46	422	TPR	0.95
374	LTG	1.48	423	TPR	6.17
375	LTG	6.52	424	TPR	0.64
376	LTG	1.70	425	TPR	1.61
377	LTG	2.63	426	TPR	8.81
378	LTG	1.45	427	VPA	57.12
379	LTG	4.75	428	VPA	101.33
380	LTG	5.39	429	VPA	52.66
381	LTG	2.86	430	VPA	74.41
382	PBT	25.23	431	VPA	66.15
383	PBT	2.68	432	ZNS	20.8
384	PBT	24.80	433	ZNS	12.8
385	PBT	12.00	434	ZNS	8.21
386	PBT	24.03	435	ZNS	8.92
387	PBT	14.82	436	ZNS	8.27
388	PBT	22.81	437	ZNS	12.63
389	PBT	10.16	438	ZNS	3.75
390	PBT	54.83	439	ZNS	19.49
391	PBT	12.42	440	ZNS	23.77
392	PBT	0.00	441	ZNS	9.24
393	PBT	14.74	442	ZNS	36.89
394	PBT	32.53	443	ZNS	6.14
395	PBT	107.11	444	ZNS	46.69
396	PGR	1.37	445	ZNS	35.73
397	PGR	6.25	446	ZNS	27.10
398	PGR	0.00	447	ZNS	26.05
399	PHT	13.11	448	ZNS	48.81
400	PHT	16.29	449	ZNS	10.81
401	PHT	8.72	450	ZNS	20.58
402	PHT	9.61	451	ZNS	5.12
403	PHT	6.34	452	ZNS	76.12
404	PHT	11.83	453	ZNS	4.84
405	PHT	6.54			
406	PHT	1.60			
407	PHT	10.74			
408	RFM	17.13			
409	RFM	13.06			
410	RFM	21.34			
411	RFM	2.79			

* AED1 column refers to AEDs requested to be tested.

- Shaded cells refer to samples with concentrations below or above the therapeutic range.

7 Gabapentin and Pregabalin Prevalence Among Prisoners in Scotland: An Insight Into Their Abuse Potential.

7.1 Introduction

In Scotland, drug and addiction services such as the Scottish Drug Forum in Glasgow and Crew in Edinburgh have raised concerns regarding gabapentin and pregabalin being misused on the street and among prisoners. A recent survey including 129 participants showed a 22 % prevalence of GBP and PGR among drug abusers in Edinburgh (109). This study gave an indication of the prevalence of these drugs. However, participants at the addiction clinics were often unregistered and would turn up without appointments, so it was not possible to determine an accurate response rate to this survey. Also, participants may not have stated all drugs that they had been using and therefore provide misleading data.

In order to evaluate the prevalence and abuse potential of AEDs among prisoners in Scotland, a study was carried out in collaboration with the Scottish Prison Service (SPS) after obtaining the required ethical approval from West of Scotland Research Ethics Service (WoSRES). Eight prisons in Scotland participated in this study and urine samples from admitted and released prisoners over a one month period were collected. The urine samples were analysed using a LC/MS/MS-QQQ method which was developed and validated for the simultaneous quantification of 21 AEDs in urine.

Although urine samples can be used only for qualitative analysis, and the presence of drugs is only indicative of previous exposure, urine has several advantages over other matrices such as blood and hair. Many drugs and their metabolites are present in higher concentrations in the urine than in the blood. Some classes of drugs also remain in the urine for days or longer following their use. Urine can be noninvasively collected, and does not require skilled personnel to perform this task (289).

This is the first prevalence study to be carried out on a large scale in Scotland to assess the prevalence and abuse potential of gabapentinoids and other AEDs using urine analysis to confirm the drug consumption.

7.2 Ethical Approval

The study was approved by the West of Scotland Research Ethics Service (WoSRES), (reference: 12/WS/0312) (Appendix 7-1). The project was given ethical approval for urine and hair sample collection. However, due to the high work load at the prison sites, SPS advised that the hair sampling would cause much interruption for the prison health officers and prisoners were unlikely to cooperate to donate their hair samples. This was also the case with urine samples initially. In order to solve this issue, they kindly suggested participating in their annual drug of abuse prevalence study in November of each year where they collect urine samples routinely from all admitted and released prisoners. Hence, hair sample analysis was stopped at this point and the project focused on urine sample analysis.

7.3 Methodology

7.3.1 Study Design

Out of the 16 prisons in Scotland, 8 prisons participated in this study; Addiwell, Perth, Barlinnie, Polmont, Low Moss, Corton Vale, Edinburgh, and Greenock (Figure 7-1).

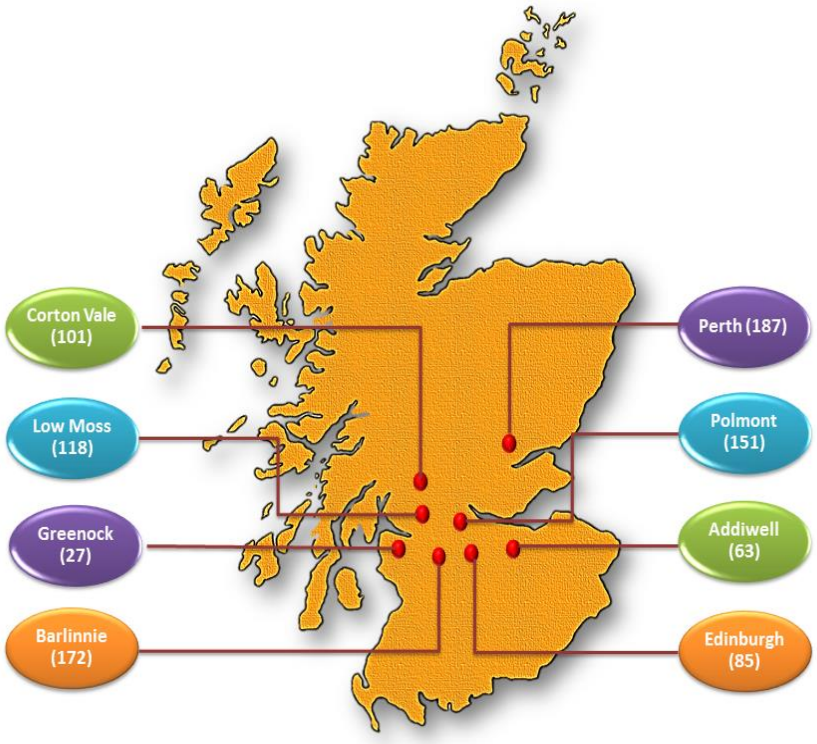


Figure 7-1: Regional Distribution and Number of Collected Samples From Participating Prisons.

All these establishments are ‘closed’ prisons with facilities in place to prevent escape. Community access is available to suitably risk assessed offenders from Barlinnie, Corton Vale, Greenock and Polmont (290).

The prisons were chosen based on the number of prisoners with the exception of Greenock prison which requested to participate in the study in spite of its small population. Participants were all prisoners, male and female, released from and admitted to the selected prisons over a one month period (November 2013).

Urine samples were initially collected by the SPS to evaluate the prevalence of other illicit drugs. All specimens and data were encoded with a serial number to protect the prisoner’s confidentiality. After the samples had been tested by the SPS, they were transferred to the department of Forensic Medicine and Science and stored at -20 °C until analysis.

7.3.2 Calibrators, Internal Standards and Quality Control Preparation

All calibrators, internal standards and quality controls were prepared in similar way to those prepared for blood, serum and plasma (see 5.2.3 and 5.2.4 Chapter 5 for details).

7.3.3 Urine Sample Preparation

On the day of analysis, the samples were left to thaw at room temperature. A 100 µL aliquot of the urine sample was transferred to a 2 mL snap top polypropylene microcentrifuge tube, 100 µL of internal standard solution and 300 µL methanol were added, vortex mixed for 30 seconds and centrifuged for 10 minutes at 10000 rpm. An aliquot of 200 µL of the supernatant was transferred to a LC vial and diluted with 500 µL of deionized water. 5 µL of the diluted supernatant was injected into the LC/MS/MS.

Since the focus of this study was gabapentin and pregabalin prevalence among prisoners, no urine hydrolysis was applied as more than 81% of gabapentin and pregabalin is excreted unchanged in urine (see 1.6.3 for AEDs metabolism and excretion).

7.3.4 Instrumentation

This was the same as described in 3.2.3.

7.3.5 Qualitative Method Validation

The method was previously validated for whole blood using the same instrument (see Chapter 3). Due to matrix change which might have an effect on the precision, accuracy, recovery and matrix effect, a revalidation was required for urine.

Before screening the urine samples, the method was qualitatively validated, initially for 21 AEDs, to determine cut offs. NA-RTG could not be analysed because it was out of stock. According to standard practices for method validation in forensic toxicology (SWGTOX, May 2013), qualitative method validation parameters are:

a) Selectivity

b) Carryover

c) Matrix effect

d) Limits of detections

Selectivity was assessed using negative case samples. *Specificity* was assessed by spiking drug-free matrix with each AED individually. Interferences were examined visually.

Assay LODs were determined for urine using 3 different sources of blank urine samples spiked with decreasing concentrations of AEDs and analyzed in duplicate for three separate runs.

Carryover was tested by injecting three blank controls after two injections of the upper limit of quantification in the calibration curve. It was evaluated by dividing the blank peak area at the expected retention time by the mean peak area of the ULOQ and multiplying by 100. No carryover is considered if the value is lower than 10%.

Recovery and Matrix effect were evaluated using the post-extraction addition approach for all 21 AEDs in urine (see 2.2.12.4 for details) (248).

7.3.6 Quantitative Method Validation of Pregabalin and Gabapentin

Due to the considerable number of gabapentin and pregabalin positive samples detected during the qualitative analysis; a linearity, precision and accuracy check for these two drugs was carried out before re-analysing the positive samples quantitatively. Linearity was assessed by analysing five separate calibration curves prepared by spiking blank blood with gabapentin and pregabalin at 8 concentrations ranging from 0.5-50 mg/L. A linear regression equation weighted 1/X was applied.

7.4 Results and Discussion

7.4.1 Qualitative Method Validation

7.4.1.1 Selectivity

No endogenous or exogenous interference was observed and none of the AEDs or their internal standards showed any interference at the retention time of the other drugs included in the method. The generated results from urine were comparable to the whole blood, serum and plasma selectivity study in the previous methods (see 3.3.1 and 5.3.3).

7.4.1.2 LODs, LLOQs and LOQs

Instrument LODs were not re-evaluated as they were previously determined (see 3.2.5). Compared to whole blood, serum and plasma, the LODs, LLOQs and LOQs for all drugs in urine were very similar to those of the other matrices in spite of the different composition and content of each matrix (see 3.3.2 and 5.3.4).

Table 7-1: Assay LOD, LLOQ and LOQ of 21 AEDs in Urine.

AEDs	LOD	LLOQ	LOQ
CBZ	0.25	0.5	0.5
CBZO	0.05	0.25	0.5
ESL	0.25	0.5	0.5
GBP	0.1	0.25	0.5
LAC	0.05	0.25	0.5
LEV	0.1	0.5	5.0
S-LC	0.1	0.25	0.5
LTG	0.25	0.5	0.5
OXC	0.05	0.1	0.05
PBT	1.0	2.5	2.5
PGR	0.5	1.0	0.5
PHT	0.5	1.0	1.0
p-HPPH	0.5	1.0	1.0
RFM	0.1	0.25	0.5
RTG	0.025	0.05	0.05
STP	0.25	0.5	0.5
TIG	0.01	0.025	0.05
TPR	0.25	0.5	0.5
VIG	0.5	1.0	5.0
VPA	2.5	5.0	5.0
ZNS	0.5	1.0	1.0

7.4.1.3 Recovery and Matrix Effect

The matrix factor and recovery results of 2 QCs (low and high) using 6 different sources of matrix are detailed in Table 7-2. Matrix factor values were acceptable for all the drugs (within ± 1.25) and ranged between 0.81 and 1.13. Recovery was greater than 80% for all the AEDs. The recovery and matrix effect results in urine were comparable to the values obtained in whole blood, serum and plasma except for retigabine. Retigabine exhibited an acceptable matrix suppression effect (MF=0.81- 0.86) in all 4 matrices and good recovery (>80) in urine, serum and plasma but less so in whole blood which was around 71 % (see 5.3.7).

Table 7-2: Recovery and Matrix Factor Values for 21 AEDs Using Low and High QCs and 6 Different Urine Sources *(n=6 per QC per Matrix).*

AEDs	QC1		QC2	
	Recovery(%)	*Matrix effect*	*Recovery(%)*	*Matrix effect*
CBZ	107	1.02±0.05	106	1.02±0.12
CBZO	91	1.04±0.05	98	1.02±0.11
ESL	89	0.82±0.29	95	0.84±0.30
LAC	93	1.04±0.04	97	1.00±0.10
LEV	92	1.01±0.05	95	1.00±0.10
LIC/SLE	87	1.01±0.06	89	1.00±0.11
LTG	109	1.03±0.03	102	1.01±0.07
GBP	104	1.13±0.07	105	1.06±0.09
PBT	83	0.87±0.08	86	0.87±0.08
PGR	98	1.03±0.04	98	1.00±0.14
OXC	105	0.89±0.30	110	0.81±0.16
PHT	88	0.99±0.05	90	1.02±0.15
p-HPPH	85	1.00±0.07	81	1.02±0.08
RFM	96	1.00±0.08	93	1.02±0.09
RTG	87	0.90±1.84	81	0.81±1.16
TIG	80	1.00±0.04	85	1.04±0.05
TPR	102	1.00±0.03	105	1.05±0.09
VIG	76	0.90±0.23	84	0.90±0.19
VPA	103	1.03±0.06	108	1.02±0.14
ZNS	92	1.03±0.06	92	1.04±0.12

7.4.1.4 Carryover

No carry over was observed for all 21 AEDs in urine. Carryover percentage after the first blank injection was 0% for all drugs except levetiracetam, valproic acid and levetiracetam which was 0.06%, 0.03% and 0.35% respectively. However, these percentage are acceptable (< 10%) and very low compared to the high concentrations used as an ULOQ (300 mg/L). Summary of the carryover results is detailed in Table 7-3.

Table 7-3: Carryover Results of 21 AEDs After Double Injections of Extracted ULOQ in Urine.

AED	Peak Area				Carryover (%)*
	Mean ULOQ (n=2)	Blank 1	Blank 2	Blank 3	
CBZ	1458085	0	0	0	0.00
CBZO	226910	0	0	0	0.00
ESL	1584816	0	0	0	0.00
GBP	355521	0	0	0	0.00
LAC	861567	0	0	0	0.00
LEV	180120	111	0	0	**0.06**
LIC/SLE	1459655	0	0	0	0.00
LTG	22188	0	0	0	0.00
OXC	12086	0	0	0	0.00
PBT	66953	0	0	0	0.00
PGR	178962	0	0	0	0.00
PHT	5679	0	0	0	0.00
HPPH	4033	0	0	0	0.00
RTG	115958	0	0	0	0.00
RFM	3787	0	0	0	0.00
STP	21644	0	0	0	0.00
TIG	32889	0	0	0	0.00
TPR	30365	0	0	0	0.00
VIG	110506	28	18	0	**0.03**
VPA	226775	799	62	0	**0.35**
ZNS	7935	0	0	0	0.00

* Carryover percentage was calculated by dividing blank 1 peak area by ULOQ Mean peak area and multiplying by 100.

7.4.2 Quantitative Method Validation of Pregabalin and Gabapentin

The calibration curves were linear with a R^2 greater than 0.998. Accuracy and precision were assessed by analysing replicates of spiked controls at 2 different concentrations (7 and 45 mg/L). Intra-day precision and bias were calculated from 3 replicates per QC in one batch. Inter-day precision and bias were determined over 5 different runs. The accuracy

values were within the acceptable range of ±15% of the nominal concentrations. The intra- and inter-day accuracy ranged from 93.8 - 104.4% for gabapentin and 96.2 - 105.3% for pregabalin. Both intra and inter-day precision values were acceptable and less than the 15%. The intra- and inter day precision values were less than 10.4% for gabapentin and less than 7.4% for pregabalin (Table 7-4).

Table 7-4: Accuracy and Precision Results of Gabapentin and Pregabalin in Urine.

AEDs	Precision				Accuracy			
	Intra-day (%) n=3		Inter-day (%) n=15		Intra-day (%) n=3		Inter-day (%) n=15	
	7 mg/L	**45 mg/L**	**7 mg/L**	**45 mg/L**	**7 mg/L**	**45 mg/L**	**7 mg/L**	**45 mg/L**
GBP	2.0	1.7	10.4	5.9	93.8	104.4	93.8	104.4
PGR	5.3	2.7	6.9	7.4	96.2	105.3	96.2	105.3

7.4.3 Demographic Data

A total of 904 urine samples were collected from the 8 prisons over a one month period (November 2013). Samples collected were from prisoners who were just admitted to or released from prison in November 2013. Due to the high workloads at the prisons, the only information provided with the samples was the sample numbers and whether they were admission or liberation samples. Hence, accurate demographic data regarding gender and age was not applicable in this study. The available data is shown in Table 7-5.

Table 7-5: Demographic Data on Selected Prisons (291, 292).

Prison	Capacity	Population[a]	Sex[b]	Age	Total	A[c]	L[d]	Facility	Area served
Addiwell	700	705	M	>21	63	33	30	Local	Lanarkshire, West Lothian
Barlinnie	1021	1256	M	>21	172	106	66	Local	West of Scotland
Corton Vale	309	221	F	>16	101	90	11	National	Whole of Scotland
Edinburgh	870	819	M&F	>21	85	25	60	Local	Edinburgh, Lothian, Borders, Kirkcaldy, Fife
Greenock[e]	255	244	M&F	>21	27	11	16	Local	West of Scotland
Low Moss	784	739	M	>21	118	100	18	Local	North Strathclyde
Perth	633	679	M	>21	187	123	64	Local	Angus, Dundee, Perth, Kinross, Fife
Polmont	760	503	M	16-21	151	102	49	National	Whole of Scotland
Total	**5332**	**5166**			**904**	**590**	**314**		

a: Prison Population number and Facility type were last updated on 21-November-2014 by SPS.

b: M=Male, F=Female. c: A=Admission. d: L=Liberation.

e: Greenock is a male prison but it held a number of female prisoners at the time of the study due to Corton Vale establishment renovation.

7.4.4 Admission vs Liberation

As shown in Table 7-6, the total AEDs prevalence for admission and liberation is similar (20% and 16% respectively). Out of 164 positive samples, 115 were admission samples (68%) compared to 49 liberation samples (32%). However, AEDs prevalence at Edinburgh prison was mainly liberation samples (Figure 7-2). This may have been due to the high number of liberation samples received over this month; 60 samples out of the total 85 samples collected were liberation samples. These results can be an indication of GBP and PGR abuse among prisoners at Edinburgh prison.

Table 7-6: Admission, Liberation AEDs Prevalence on Selected Prisons.

Prison	Positive Samples	Prevalence (%)	Admission		Liberation	
			Positive	(%)	Positive	(%)
Addiwell	16	25	12	44	4	13
Barlinnie	16	9	11	10	5	8
Corton Vale	28	28	25	28	3	27
Edinburgh	21	25	3	12	18	30
Greenock	1	4	1	9	0	0
Low Moss	23	22	21	21	2	11
Perth	51	27	38	31	13	20
Polmont	8	5	4	4	4	8
Total	164	18	115	20	49	16

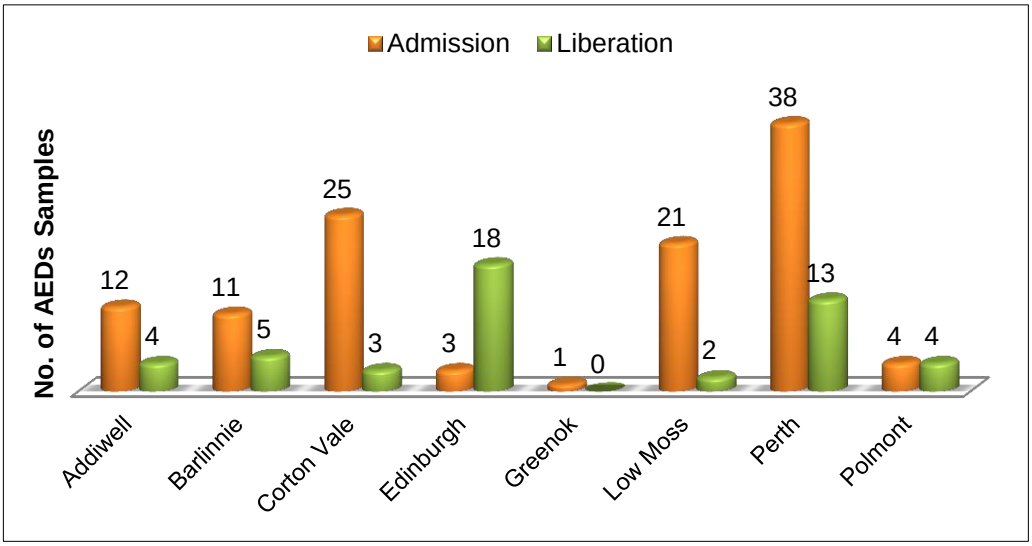


Figure 7-2: AEDs Prevalence Among Admitted and Released Prisoners.

7.4.5 AEDs Prevalence per Prison

The results of the analysis found 164 of the 904 samples to be positive for at least one AED (18%). Five of the prisons had prevalence higher than 20% (Table 7-6).

Corton Vale is Scotland's only all-female prison and had the highest AEDs prevalence at 28%, followed by Perth (27%), Addiwell (25%), Edinburgh (25%) and Low Moss (22%). Barlinnie is Scotland's largest establishment and holds all categories of prisoners. It is known to be overcrowded with prisoners sharing cells meant for individual use. It is the largest dispenser of heroin substitute, methadone in Western Europe with drug use in over 80% of its population (18). However, AEDs prevalence was only 9% at this prison.

Polmont is Scotland's national holding facility for young offenders aged between 16 - 21 years. It showed a very low prevalence of AEDs (5%) among this age group. This can either be seen as a sign of their low popularity among adolescents or an indication of their low prescription rate among this group of people.

Finally, Greenock is a small establishment which accommodates a small number of prisoners for a range of management and operational reasons. It participated with 27 samples of which one was positive for GBP. More details of all AEDs detected at each prison are shown in Table 7-7.

Table 7-7: Number of Each AED Detected at Each Prison.

Prison	GBP		PGR		LEV		LTG		VPA		VIG	
	A*	L*	A	L	A	L	A	L	A	L	A	L
Addiwell	11	3	1	1	-	-	-	-	-	-	-	-
Barlinnie	10	3	1	2	-	-	-	-	-	-	-	-
Corton Vale	20	0	3	3	-	-	1	-	1	-	-	-
Edinburgh	3	9	-	6	-	1	-	-	-	-	-	2
Greenok	1	-	-	-	-	-	-	-	-	-	-	-
Low Moss	1	16	4	-	1	-	-	-	1	-	-	-
Perth	30	8	3	5	-	-	2	-	1	-	-	-
Polmont	3	-	-	3	1	1	-	-	-	-	-	-
	79	**39**	**12**	**20**	**2**	**2**	**3**	**0**	**3**	**0**	**0**	**2**

*A: Admission, L: Liberation

7.4.6 AEDs Prevalence by Drug

The high prevalence of AEDs is mainly due to GBP and PGR on their own or in combination (Figure 7-3). GBP was identified in 118 samples out of the 164 positive samples (68%) and PGR in 32 samples (13%). Other AEDs detected were levetiracetam (LEV) and vigabatrin (VIG); 4 samples each. Lamotrigine (LTG) and valproic acid (VPA) were positive in 3 samples each. Carbamazepine (CBZ) and topiramate (TPR) were found in 2 and 1 samples respectively.

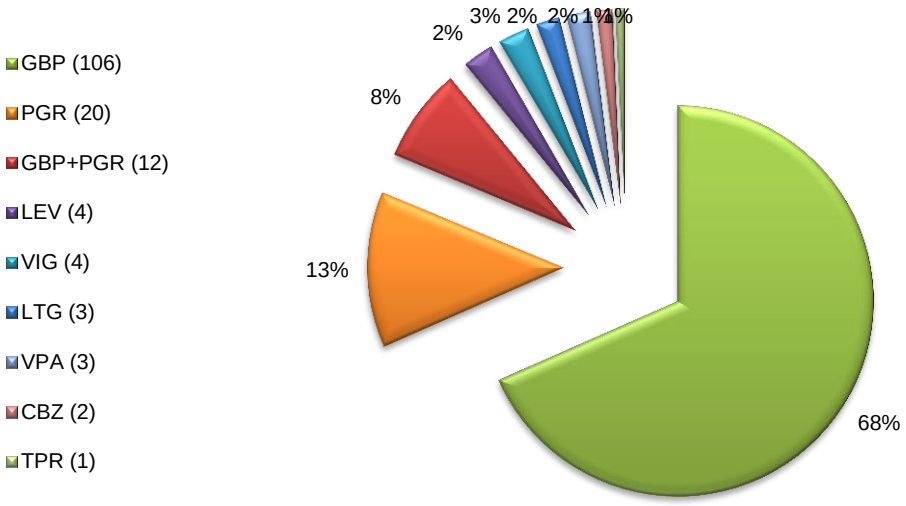


Figure 7-3: AEDs Prevalence per Drug.

Out of the 164 samples, only 15 samples were positive for 2 AEDs (9%). Interestingly, 12 of these specimens contained both GBP and PGR; 8 admission and 4 liberation samples (8%). Other drug combinations found were GBP with LTG (1 sample), GBP with TPR (1 sample) and LEV with VPA (1 sample).

Since neuropathic pain treatment guidelines recommend combination therapy using drugs with different mechanisms of action, it seems unlikely that practitioners would routinely prescribe both gabapentinoids together (293, 294). The presence of both drugs may instead represent a change in therapy from one agent to the other. The half-lives of gabapentin (t½ = 5.9 h) (295) and pregabalin (t½ = 4.6–6.8 h) (296) are relatively short. However, because sudden termination may trigger withdrawal symptoms, changing therapy would typically require reducing the dose of the initial drug while escalating the dose of the replacement one over at least 5-7 days. In such cases, patients could test positive for both drugs. Alternatively, presence of both drugs might indicate medication dependence or abuse. PGR and GBP concentrations in these 12 samples are presented in Table 7-8.

Sample 10 had high concentrations of GBP and PGR (82, 141 mg/L respectively) which might be a case of abuse.

Table 7-8: GBP and PGR Concentrations in Samples Containing Both Drugs.

Sample No.	Sample Type	GBP (mg/L)	PGR (mg/L)
1	A	2.2	25.1
2	L	3.0	1.1
3	L	8.1	14.2
4	A	10.5	128.7
5	L	14.1	2.2
6	L	18.1	2.7
7	A	36.4	438.2
8	A	42.7	0.8
9	A	66.9	0.6
10	A	81.7	141.1
11	A	93.7	2.6
12	A	170.8	15.2

7.4.7 GBP and PGR Concentration Frequencies

As shown in Figure 7-4 and Figure 7-5, urine concentrations of GBP and PGR varied across a broad concentration range. The concentrations ranged from 0.4-1100 mg/L (mean: 61.6 ± 129 mg/L, median: 15 mg/L) for GBP and from 0.4-440 mg/L (mean: 59.9±114 mg/L, median: 7.3 mg/L) for PGR.

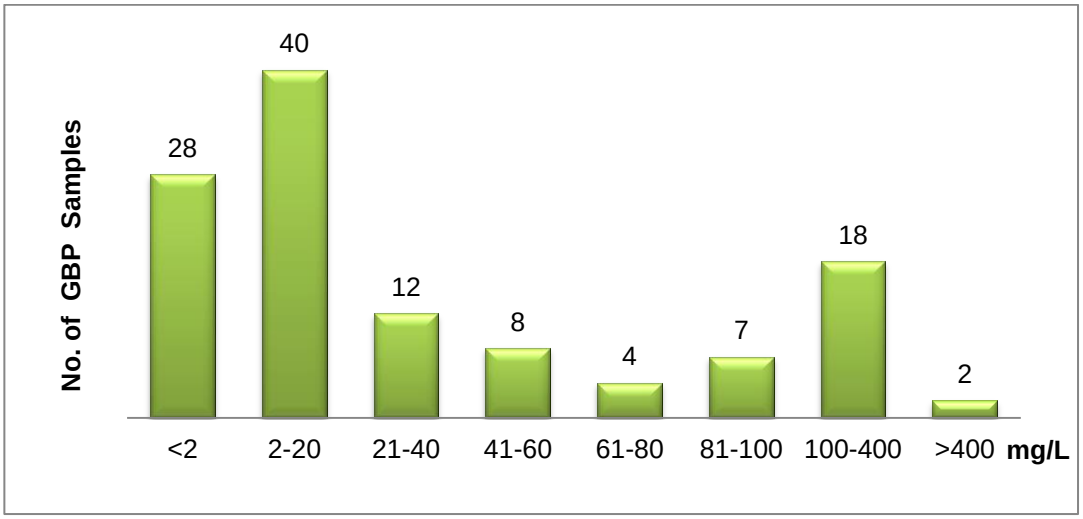


Figure 7-4: GBP Concentration Ranges in 118 Positive Samples.

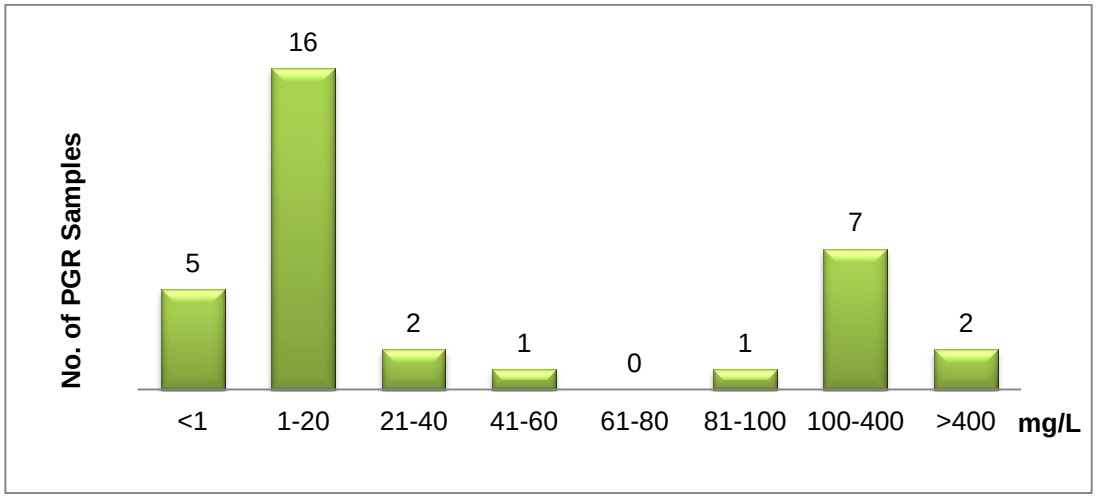


Figure 7-5: PGR Concentration Ranges in 32 Positive Samples.

The median concentrations of GBP were two fold greater than pregabalin. This is generally consistent with the relative potency of these drugs. The recommended dose of GBP is 900–1800 mg/day and its therapeutic range varies between 2.2 to 6.1 mg/L, whereas the recommended dose of PGR is 150–600 mg/day with therapeutic range 1.3 to 4.9 mg/L (137). Both drugs are not metabolized, not bonded to plasma protein and are eliminated unchanged by the kidneys; 81% for GBP and 92% for PGR (137). Urine concentrations were reported to range from 2.5 to 35345 mg/L for GBP and 2.5 to 6892 mg/L for PGR among pain clinic patients but it was unknown whether all patients were prescribed these two drugs or not (297). In this study, 20 GBP samples (17%) and 10 PGR samples (31%) had urine concentrations 5-50 fold higher than the median values of both drugs. Since no medication history was available for interpretation of results, it was difficult to determine whether these high concentrations were due to prescribed doses of medication or misuse among the prison population.

7.4.8 Illicit Drugs Prevalence in Positive AEDs Samples

The urine samples were initially screened by the SPS for drugs of abuse using urine dipstick analysis. Drugs tested by SPS were amphetamines, barbiturates, benzodiazepines, buprenorphine, cannabis, cocaine, methadone, methamphetamines and opiates. AEDs positive samples were compared with SPS results in order to investigate other illicit drugs associated with AEDs. Information provided by SPS was sample number, screening results and whether the drug was prescribed or not. All non-prescribed drugs were considered abused by SPS.

Unfortunately, only 117 out of the 164 AEDs samples were matched with the SPS database due to the variation between their numbering system and the current study numbering system. 81 % of the matched samples were positive for at least one illicit drug. It was obvious that the number of illicit drugs detected among admitted prisoners (n=93) was higher than the liberated ones (n=24) as illustrated in Figure 7-6.

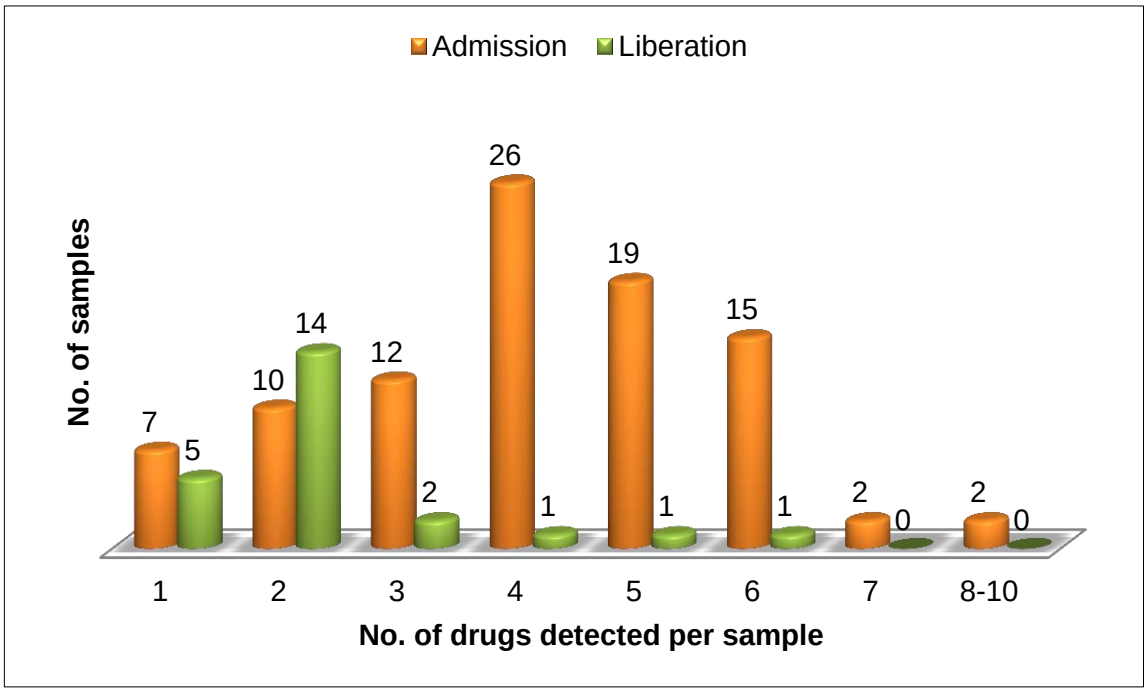


Figure 7-6: Number of Drugs Detected in Samples Among Admitted and Released Prisoners.

The most frequently detected drugs along with AEDs were benzodiazepines (67%), opiates (57%) and cannabis (47%) including prescribed and non-prescribed samples as shown in Figure 7-7. The majority of these samples contained non-prescribed drugs. For instance, benzodiazepines were non-prescribed in 78 samples (61%) and opiates were also misused in 63 samples (54%) whereas all 55 cannabis samples were non-prescribed (47%). Methadone was positive in 51 samples (44%) of which 31 samples were abuse cases (26%). Cocaine and buprenorphine were also detected in 18 and 17% of the samples respectively as abuse use, whereas amphetamines, methamphetamines and barbiturates were only found in 4% of the AEDs samples and were all abuse cases. Details of drugs detected in samples are presented in Appendix 7-2.

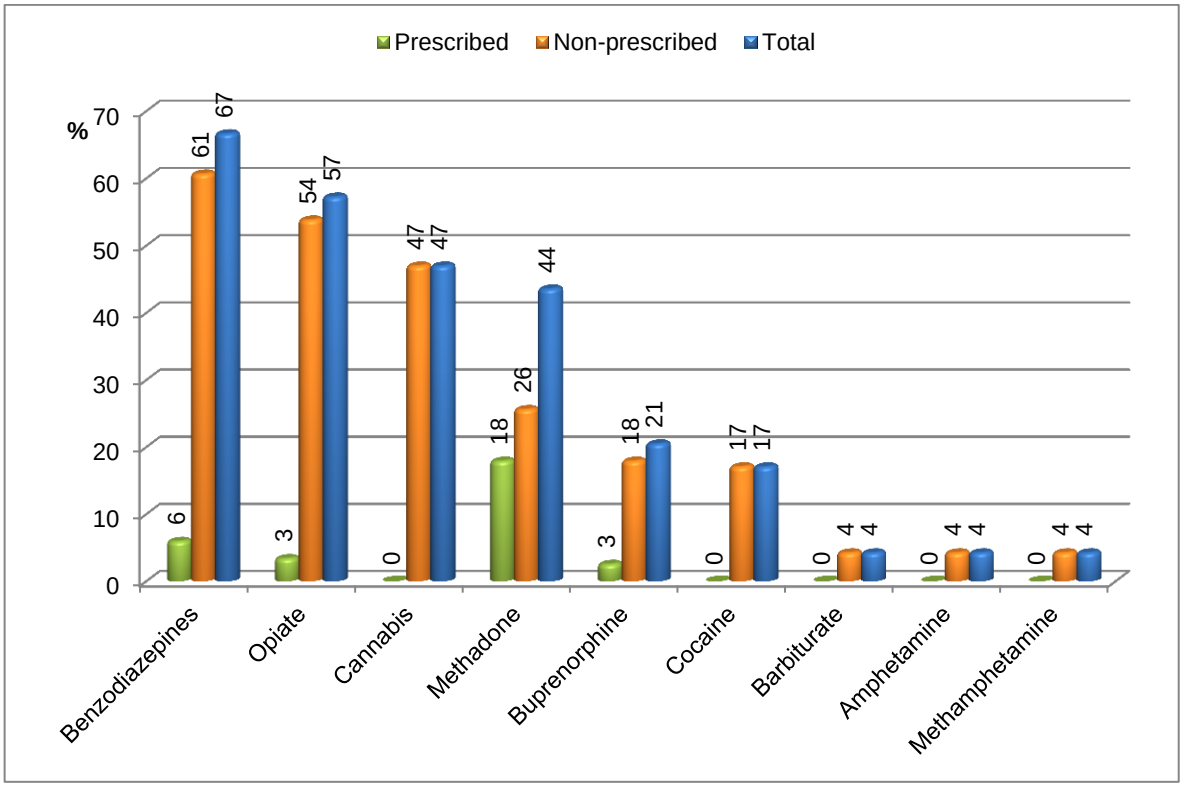


Figure 7-7: Prescribed and Non-prescribed Illicit Drugs Associated With AEDs.

7.5 Study Limitation

The major limitation of the study is the lack of information provided along with each sample regarding age, sex and medical history which does not allow differentiation between prescribed and abused AEDs. In order to obtain further information to distinguish between prescribed and abuse cases, the prison GP leads were contacted. Unfortunately due to time restrictions and high workloads, only one prison lead responded. In the response received, it was mentioned that AEDs prevalence in this study is not surprising and that they are aware of the drug abuse issue among prisoners.

The difficulty in obtaining further information from medical records is largely due to the prescription system used within the prisons. The prescribing system is not computerised and paper files are used to record prisoners' medical history. Finding the relevant information was therefore seen as time consuming. Additionally, the records may not be complete as prisoners may not state all the drugs that they have been using before their admission and give misleading data.

7.6 Conclusion

The aim of this study was to evaluate the prevalence and abuse potential of (AEDs) among prison populations. Eight prisons in Scotland participated in this study and urine samples from admitted and released prisoners over a one month period were collected. Urine samples were collected and analysed using a LC/MS/MS-QQQ method which was developed and validated for the simultaneous quantification of 21 AEDs in urine. In spite of the lack of the medical history, the study results were comparable to the survey conducted in Edinburgh but on a larger scale (8). The study shows a high prevalence of AEDs (18%) largely due to GBP and PGR on their own or in combination with other drugs of abuse. Gabapentin was identified in 118 samples (13%) and pregabalin in 32 samples (4%). Interestingly, 12 samples contained both drugs (7%). The concentrations ranged from 0.4-1100 mg/L (median: 15 mg/L) for gabapentin and from 0.4 - 440 mg/L (median: 7.3 mg/L) for pregabalin. Four samples were found to be higher than 400 mg/L. These concentrations are at least 20 times above the median concentrations and it is likely that those individuals took higher doses than recommended. Other AEDs detected were levetiracetam (4 samples), vigabatrin (4 samples), lamotrigine (3 samples), valproic acid (3 samples), carbamazepine (2 samples) and topiramate (1 sample). The majority of AEDs positive samples (81 %) also contained at least one illicit or non-prescribed drug. Benzodiazepines, opiates and cannabis were the most frequently found illicit substances with a percentage of 61, 54 and 47 % respectively. Other illicit drugs found in positive AEDS samples were methadone (26%), cocaine (18%), buprenorphine (17%), amphetamines (4%), methamphetamines (4%) and barbiturates (4%). This study shows a high prevalence of AEDs largely due to gabapentin and pregabalin on their own or in combination with other drugs of abuse. Clinicians and prison GP leads should be aware of the high prevalence of gabapentinoids and their abuse along with other drugs of abuse.

8 Simultaneous Analysis of 16 AEDs in Head Hair Using LC/MS/MS

8.1 Introduction

Drug analysis in hair has multiple applications in clinical and forensic toxicology. In forensic cases, hair represents the best alternative choice for analysis when other biological samples are not available, for example, due to sample degradation or late reporting of an incident(176), or when the history of drug use is required, for example, in therapeutic drug monitoring cases where drug taking behaviour may affect the quality of a patient's life and may be the reason for unexplained deaths like SUDEP (67). Only a few papers have been published regarding conventional AED analysis in hair. These have covered the drugs carbamazepine, valproic acid, phenobarbital, phenobarbitone, phenytoin and lamotrigine (167, 172, 174, 298). One paper was found regarding oxcarbazepine (171), lamotrigine (166) and most recently a paper was published regarding pregabalin in hair in 2013 (170).

The aim of this project was to optimize the best extraction conditions for 16 AEDs including carbamazepine and its metabolite carbamazepine-10,11-epoxide, eslicarbazepine acetate, gabapentin, lacosamide, lamotrigine, levetiracetam, oxcarbazepine, pregabalin, phenytoin and its metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin, topiramate, tigabine, valproic acid, vigabatrin and zonisamide. Six different digestion conditions and three clean up procedures were compared. The method was then qualitatively validated using the most efficient extraction for all AEDs together. Finally, two case samples from two donors who were known to be prescribed 4 AEDs; carbamazepine, gabapentin, lamotrigine for sample 1 and valproic acid for sample 2, were tested using the validated method. No more samples were available at the time of the project due to sampling issues at the prison sites (see 7.2) and ethical approval being rejected to collect hair samples from known epileptic patients.

8.2 Materials and Methods

Method development was carried out using hair samples obtained from persons known not to have used any drugs being investigated in this study. The hair samples were prepared and spiked with the appropriate standards as described in the following sections.

8.2.1 Materials

These were the same as those described in 2.2.1.

8.2.2 Preparation of Hair Standards

8.2.2.1 Wash and Cut Procedure

In order to remove grease and cosmetic preparations, the hair samples were washed using deionized water twice, then dichloromethane (DCM) twice. At each wash stage, the appropriate solvent was added to completely immerse the hair samples and sonicated for 3 minutes at room temperature. The solvent was then decanted off and the wash step repeated. After the last hair wash with DCM, samples were dried overnight in an oven at 40°C. After the samples were washed and dried, hair was finely cut into 1-2 mm pieces using scissors and stored at room temperature for later use.

8.2.2.2 Hair Spiking Procedure

The cut hair was spiked with a mixed standard of 16 AEDs prepared in methanol. To prepare a target concentration of 1 ng/mg, 500µL of the standard at a concentration of 1 mg/L was added to 500 mg of hair sample in a glass vial. A 1 ml volume of methanol was added to ensure that all the hair was immersed. The vial was sealed and sonicated for 1 hour at 40 °C. It was then opened and the hair was allowed to dry at room temperature. This spiked hair was used for extraction optimization. Internal standards were GBP-D_{10}, TUB and CBZ-DiOH at a concentration of 10 mg/L in methanol and added to each sample vial to attain a concentration of 1 ng/mg. All hair samples were stored at room temperature.

Although the use of spiked hair is not ideal, since most of the drug will be on the surface of the hair and not incorporated into the hair, it can give an indication of the effect the extraction conditions will have on the drugs. However, to truly assess extraction recovery of an incorporated drug from hair, authentic samples should be used. Sufficient samples were not available to assess this.

8.2.2.3 Preparation of Calibrators and Quality Control

Stock solutions for 16 AEDs were prepared in methanol at 1 mg/mL for each drug individually. The solutions were further diluted to obtain two mixed working solutions at 100 ng/mL and 10 ng/mL used to spike the hair. Seven hair samples (20 mg each) were

spiked at increasing concentrations from 0.05 to 2 ng/mg as given in Table 8-1. Volumes were completed to 1 mL with methanol. This calibration was used to calculate AED concentrations detected during the qualitative analysis.

Table 8-1: Preparation Procedure of Hair Calibration Curve (Sample Size: 20 mg).

Calibrator (ng/mg)	Volume of Stock 1 (10 ng/mL), (µL)	Volume of Stock 2 (100 ng/mL), (µL)	MeOH (µL)
0.05	100		900
0.1	200		800
0.2	400		600
0.4		80	920
0.8		160	840
1.6		320	680
2		400	600

Two quality controls (QCs) were prepared at concentrations of 0.5 and 1 ng/mg as follows (Table 8-2). For the 1 ng/mL concentration, 500 µL of QC stock standard at a concentration of 1 mg/L was added to 500 mg of hair in a glass vial. For the 0.5 ng/mL concentration, 250 µL of 1 mg/L was added to 500 mg of hair. A 1 ml volume of MeOH was added to ensure that all hair was immersed. The vial was sealed and sonicated for 1 hour at 40°C. The vial was then opened and the hair was allowed to dry at room temperature.

Table 8-2: Preparation Procedure of Hair Quality Controls (Sample Size: 20mg).

QCs (ng/mg)	Sample size (mg)	Volume of Stock 3 (1mg/L), (µL)	MeOH (µL)
0.5	500	100	900
1	500	200	800

8.2.3 Instrumentation

This is the same as that described in 2.4.3.

8.2.4 Hair Extraction Optimization

Six different digestion methods were compared for 16 AEDs. These methods were previously reported or modified from the literature and applied on AEDs or on other groups of drugs (169-172, 299). Methods compared were:

a) Neutral digestion using MeOH on its own.

b) Basic digestion using basic methanol (MeOH: 25% NH_4OH) or alkaline digestion (0.1M NaOH and 1M NaOH) followed by liquid-liquid extraction (LLE).

c) Acidic digestion using acidic methanol (MeOH: trifloroacetic acid (TFA)) or 0.1M HCl followed by LLE.

For each extraction, spiked hair samples (20 mg each at 1 ng/mg concentration) were prepared in triplicate and injected in duplicate (n = 6). Samples were immersed in 1 ml solvent and sonicated for 2 hours at 60 ºC except 1M NaOH which was sonicated for only 30 minutes at 60 ºC. Samples after methanolic extraction were spiked with 100 µL internal standard 10 mg/L, evaporated and injected directly after reconstitution with 20% methanol in water, whereas strong acidic and alkaline digestions which may dissolve the LC column silica had to undergo liquid-liquid extraction with DCM before being injected into the LC/MS/MS. After LLE, samples were evaporated, reconstituted with 200 µL of 20% methanol and transferred to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS. Extraction efficiency of these methods was evaluated by comparing the recoveries.

In order to calculate the recovery, six non-extracted standards at the same concentration (1 ng/mg) were prepared at the same time. A volume of 20 µL of 1 mg/mL standard solution and 100 µL internal standard 10 mg/L were evaporated, reconstituted with 200 µL of 20 % methanol in water. Recovery was calculated using the following equation:

$$Recovery = \frac{\text{Extracted standard peak area /IS peak area ratio}}{\text{Non\_extracted standard peak area/IS peak ratio}} * 100$$

Detailed procedures of each digestion and extraction are as follows.

8.2.4.1 Methanolic Extraction

Three spiked hair samples (20 mg each) were extracted with 1 ml methanol for 2 hours under sonication at 60 °C. The supernatant was then transferred to a 7 ml glass vial. The hair was washed twice with 0.5 ml methanol and both fractions were added to the first extract. A 100 µL of the 10 mg/L internal standard solution was added. The supernatant was evaporated to dryness under a stream of N_2 and the residue reconstituted in 200 µl of

20 % methanol (in water) and transferred to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS.

8.2.4.2 Basic Methanolic Extraction [MeOH: 25% NH_4OH (20:1)]

Three spiked hair samples (20 mg each) were extracted with 1 ml of MeOH: 25% NH_4OH (20:1) for 2 hours under sonication at 60 °C. The supernatant was then transferred to a 7 ml glass vial. The hair was washed twice with 0.5 ml MeOH: 25% NH_4OH (20:1) and both fractions were added to the first extract. A 100 µL of the 10 mg/L internal standard solution was added. The supernatant was evaporated to dryness under a stream of N_2 and the residue reconstituted in 200 µL of 20% methanol and transferred to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS.

8.2.4.3 Acidic Methanolic Extraction [MeOH: TFA (50:1)]

Three spiked hair samples (20 mg each) were extracted with 1 ml of MeOH: TFA (50:1) for 2 hours under sonication at 60 °C. The supernatant was then transferred to a 7mL glass vial. The hair was washed twice with 0.5 mL MeOH: TFA (50:1) and both fractions were added to the first extract. A 100 µL of the 10 mg/L internal standard solution was added. The supernatant was evaporated to dryness under a stream of N_2 and the residue reconstituted in 200 µL of 20% methanol and transferred to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS.

8.2.4.4 0.1 M HCl Digestion Followed by LLE with DCM

Three spiked hair samples (20 mg each) were digested with 1 mL of 0.1 M HCl for 2 hours under sonication at 60 °C. The supernatant was then transferred to a 7ml glass vial. 1mL of 0.1 M NaOH was added followed by 3 mL of 0.1 M phosphate buffer (pH 6). This solution was extracted with 5 mL of DCM by vortex mixing for 1 minute followed by 10 minutes centrifuging at 3000 rpm. The lower organic layer was evaporated to dryness after adding 100 µL of the 10 mg/L internal standard solution and reconstituted in 200 µL of 20% methanol and transferred to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS.

8.2.4.5 0.1 M NaOH Digestion Followed by LLE with DCM

Three spiked hair samples (20 mg each) were digested with 1 mL of 0.1 M NaOH for 2 hours under sonication at 60 °C. The supernatant was then transferred to a 7 mL glass vial.

1 mL of 0.1M HCl was added followed by 3 mL of 0.1 M phosphate buffer (pH 6). This solution was extracted with 5 mL of DCM by vortex mixing for 1 minute followed by 10 minutes centrifuging at 3000 rpm. The lower organic layer was evaporated to dryness after adding 100 µL of the 10 mg/L internal standard solution and reconstituted in 200 µL of 20 % methanol and transferred to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS.

8.2.4.6 1 M NaOH Digestion Followed by LLE with DCM

Three spiked hair samples (20 mg each) were immersed in 1 mL of 1M NaOH for 30 minutes under sonication at 60 °C. The hair was filtered-off and washed twice with 0.5 mL of 1 M NaOH and both fractions were added to the first extract. This solution was extracted with 2 mL of dichloromethane by vortex mixing for 1 minute followed by 10 minutes centrifuging at 3000 rpm. The lower organic layer was evaporated to dryness after adding 100 µL of the 10 mg/L internal standard solution and reconstituted in 200 µL of 20% methanol and transferred to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS.

8.2.5 Clean Up Optimization

A cleaning step was required to remove the digested hair before the sample could be injected into the LC/MS/MS. In order to get cleaner samples, two cleaning procedures were evaluated; LLE and sample filtration. Three different solvents; DCM, ethyl acetate and 9:1ethyl acetate with isopropanol (IPA), were assessed for liquid-liquid extraction to compare the effect of the different solvents on recovery.

Sample filtration is a very common technique for sample clean up in LC/MS/MS. The following filters have different types of membranes (300):

a) Hydrophilic membranes with water affinity such as GHP, PES, Nylon, or PVDF membranes for filtering aqueous samples.

b) Hydrophobic membranes with affinity to aggressive organic solvents such as PTFE, making them ideal for gases and organic solvents.

c) Universal membranes such as hydrophilic polypropylene (GHP) membrane which is compatible with both aqueous and organic applications.

Most of these membranes with the Acrodisc® syringe filter are low in biomolecule binding, less than 1%. Acrodisc® filter structure is presented in Figure 8-1. Due to the variety of chemical structures of AEDs in this study, a universal GHP (0.45 µm, 13 mm) filter was used.

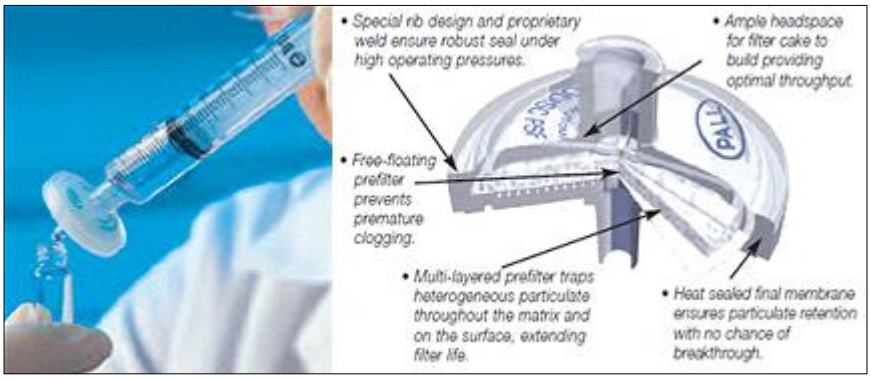


Figure 8-1: Filtration Technique Used in Hair Sample Clean-Up (300).

Analyte peak area/internal standard peak area ratio was used and recoveries were calculated as detailed previously in 8.2.4. All clean-up methods were applied on the methanolic extract as outlined below.

8.2.5.1 Methanolic Extraction Followed by Sample Filtration

Three spiked hair samples (20 mg each) were extracted with 1 mL methanol for 2 hours under sonication at 60 °C. The supernatant was then transferred to a 7 mL glass vial. The hair was washed twice with 0.5 mL methanol and both fractions were added to the first extract. A 100 µL of the 10 mg/L internal standard solution was added. The supernatant was evaporated to dryness under a stream of N_2 and the residue reconstituted in 200 µL of 20% methanol. The reconstitution then was filtered using Acrodisc® 13 mm syringe filters with 0.45 µm GHP membrane before transferring to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS.

8.2.5.2 Methanolic Extraction Followed by LLE with DCM

Three spiked hair samples (20 mg each) were extracted with 1 mL methanol for 2 hours under sonication at 60 °C. The supernatant was then transferred to a 7 mL glass vial. The hair was washed twice with 0.5 mL methanol and both fractions were added to the first extract. The supernatant was evaporated to dryness under a stream of N_2 and the residue was reconstituted with 2 mL of 0.1 M phosphate buffer (pH 6). This solution was extracted with 2 mL of DCM by vortex mixing for 1 minute followed by 10 minutes centrifuging at

3000 rpm. The lower organic layer was evaporated to dryness after adding 100 µL of the 10mg/L internal standard solution and reconstituted in 200 µL of 20 % methanol and transferred to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS.

8.2.5.3 Methanolic Extraction Followed by LLE with Ethyl Acetate

Three spiked hair samples (20 mg each) were extracted with 1 mL methanol for 2 hours under sonication at 60 °C. The supernatant was then transferred to a 7 mL glass vial. The hair was washed twice with 0.5 mL methanol and both fractions were added to the first extract. The supernatant was evaporated to dryness under a stream of N_2 and the residue was reconstituted with 2 mL of 0.1 M phosphate buffer (pH 6). This solution was extracted with 2 mL of ethyl acetate by vortex mixing for 1 minute followed by 10 minutes centrifuging at 3000 rpm. The upper organic layer was evaporated to dryness after adding 100 µL of the 10 mg/L internal standard solution and reconstituted in 200 µL of 20 % methanol and transferred to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS.

8.2.5.4 Methanolic Extraction Followed by LLE with Ethyl Acetate/IPA (9:1)

Three spiked hair samples (20 mg each) were extracted with 1 mL methanol for 2 hours under sonication at 60 °C. The supernatant was then transferred to a 7 mL glass vial. The hair was washed twice with 0.5 mL methanol and both fractions were added to the first extract. The supernatant was evaporated to dryness under a stream of N_2 and the residue was reconstituted with 2 mL of 0.1 M phosphate buffer (pH 6). This solution was extracted with 2 mL of ethyl acetate: IPA (9:1) by vortex mixing for 1 minute followed by 10 minutes centrifuging at 3000 rpm. The upper organic layer was evaporated to dryness after adding 100 µL of the internal standard solution and transferred to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS.

8.2.6 Investigation Into Extraction Conditions

The effects of incubation duration and temperature on the extraction yield were investigated. The effect of the use of GHP filters on recovery was also assessed.

8.2.6.1 Incubation Duration and Temperature

Samples were incubated using a water bath sonicator. Four conditions were evaluated to investigate the incubation duration and temperature effect on recovery as illustrated in Table 8-3. These conditions were assessed using 3 different digestion procedures; methanol on its own, basic methanol with 25% NH_4OH and acidic methanol with TFA.

Table 8-3: Incubation Duration and Temperature Conditions.

Group	Incubation Temperature	Incubation Duration	Following Step
A	40 ºC	2 Hr	Sample filtered directly and injected.
B	60 ºC	2 Hr	Sample filtered directly and injected.
C	40 ºC	1 Hr	Sample left overnight before filtration
D	60 ºC	1 Hr	Sample left overnight before filtration

Six spiked hair samples (20 mg each) were prepared for each group method. A volume of 1 mL digestion solvent (MeOH, MeOH:TFA or MeOH:25% NH_4OH) was added then incubated as illustrated in Table 8-3. After incubation, group A and B supernatants were transferred to a 7 mL glass vial. The hair was washed twice with 0.5 mL methanol and both fractions were added to the first extract. A 100 µL internal standard solution 10 mg/L was added. The supernatant was evaporated to dryness under a stream of N_2 and the residue reconstituted in 200 µL of 20 % methanol. The reconstitution was filtered using a Acrodisc® 13 mm syringe filter with 0.45 µm GHP membrane before transferring to a LC vial. A 15 µL aliquot of the reconstituted solution was injected and analysed by LC/MS/MS. Groups C and D were left overnight before their supernatants were transferred to 7 mL glass vials and these then followed the same procedure as groups A and B. Analyte peak area/internal standard peak area ratios and recoveries were calculated as detailed previously in 8.2.4.

8.2.6.2 Sample Filtration Effect on Recovery

In order to evaluate the filtration effect on recovery two sets of six hair samples were prepared. Set 1 was prepared as detailed in 8.2.4.1 without filtration. Set 2 was prepared and filtered as outlined in 8.2.5.1. A set of non-extracted standards was prepared and recoveries were calculated as outlined before in 8.2.4.

8.2.7 Qualitative Method Validation

The method was qualitatively validated according to the standard practices for method validation in forensic toxicology (SWGTOX, May 2013) for hair (13). (See 7.3.5)

Selectivity was investigated by analysing drug-free hair samples from 10 different individuals. No positive detection of any analyte of interest should be found. *Specificity* was assessed by spiking drug-free matrix with each AED individually.

Recovery and Matrix effect were evaluated in hair samples from 10 individuals using the post-extraction addition approach for all 16 AEDs in hair using three sets of samples (see 2.2.12.4). Set 1 was non-extracted standards prepared in mobile phase and injected directly without any treatment. Set 2 was prepared by spiking 6 blank hair samples (20 mg each) with standards and internal standard solutions at the beginning before digestion. Set 3 was prepared by spiking 6 blank hair samples (20 mg each) with standards and internal standard solutions after digestion/extraction but before evaporation and filtration. Both sets 2 and 3 had to undergo the filtration step due to the dirty samples resulting from the hair digestion. After analysing the samples, the peak area was used to calculate the recovery and matrix effect as detailed previously in 2.2.14.2.

Carryover was tested by injecting three blank controls after two injections of a high concentration (ULOQ = 2 ng/mg). It was evaluated by dividing the blank peak area at the expected retention time by the mean peak area of the high ULOQ and multiplying by 100. No carryover is considered if the value is lower than 10%. Carryover percentage was calculated by dividing blank 1 peak area by ULOQ mean peak area and multiplying by 100.

8.2.8 Case Samples

As mentioned previously in order for any analytical method to be fully validated, authentic samples have to be analysed. However, only two samples were available at the time of this project from two different donors due to the difficulties in obtaining authentic hair samples (see 7.2).

Sample 1 was a shaved black beard sample kindly donated from a 60 year old male who was on regular lamotrigine, gabapentin and carbamazepine prescription (chronic use). The prescribed doses were not provided.

Sample 2 was a brown head hair sample donated from a 39 year old male who was known to be prescribed valproic acid as a chronic treatment. The prescribed dose was not provided.

Case hair samples were initially analysed qualitatively, then an extracted calibration curve was prepared in hair for positively detected AEDs; carbamazepine and its metabolite carbamazepine 10, 11 epoxide, gabapentin, lamotrigine and valproic acid which were known to be prescribed for the tested samples .

Linearity, precision and accuracy were carried out before analysing the samples quantitatively. Linearity was assessed by spiking blank hair at 7 concentrations ranging from 0.05-2 ng/mg. A linear regression equation weighted 1/X was applied.

Accuracy and precision were assessed by analysing 6 replicates of spiked controls at 2 different concentrations (0.5 and 1 ng/mg). The method was not fully validated as a quantitative analysis due to instrument down time and limited time available.

8.3 Results and Discussion

8.3.1 Hair Extraction Optimization

Recoveries of six different digestion methods were compared and results are presented in Figure 8-2.

The methanolic digestions either alone or with weak acid (TFA) or weak base (NH_4OH) attained acceptable recoveries with almost all 16 AEDs including the amphoteric drugs, GBP, PGR and VIG. Recoveries were higher than 65% for all AEDs except VIG, OXC, LTG and TIG. LTG and TIG recoveries ranged between 35 - 60% whereas VIG and OXC were between 0 and 43%. Although VIG recovery was very low between 20 to 40%, the drug was still detectable compared to other digestion procedures where its recovery did not exceed 5%. OXC and CBZO have very similar structures (see 2.3.5) and both exhibited poor recovery with acidic methanol digestion (< 10 %). Strong acidic and basic digestions showed low recoveries (< 60 %) with most AEDs especially the group of amphoteric drugs with recovery less than 5%. CBZ and LAC were the only two drugs that gave a good recovery (> 55%) with all methods regardless of the pH used, whereas OXC and VIG exhibited low recovery with all digestion methods used (5 - 40 %). VPA recovery was higher than 60 % for all methods except acidic digestion and 1M NaOH digestion where its

recovery was lower than 10%. Similarly, LEV showed a recovery lower than 25 % with all strong acidic and basic digestions followed by LLE. Hence, methanolic extraction was the method of choice for hair digestion of AEDs samples in the following sections.

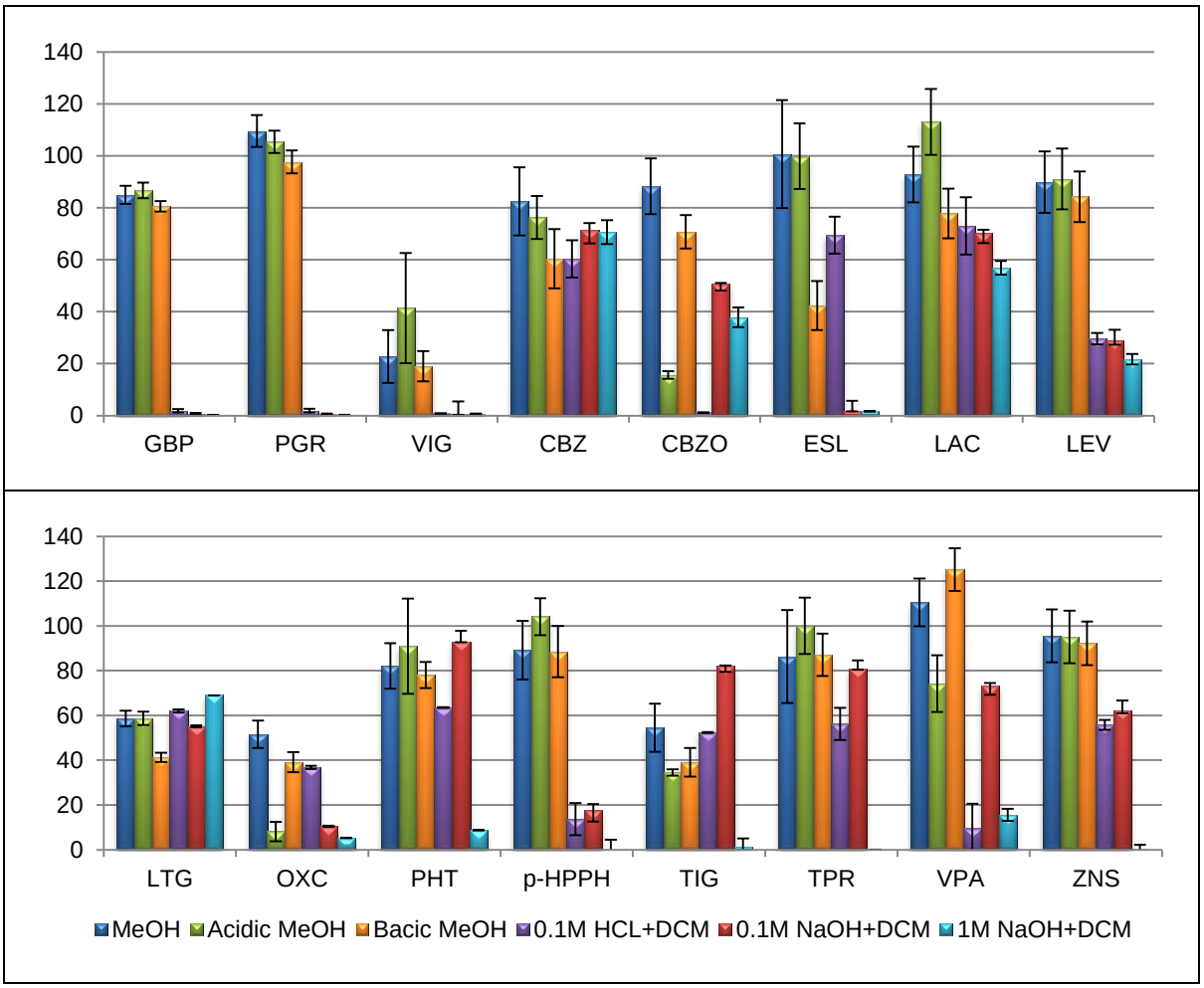


Figure 8-2: Recovery Comparison of Six Hair Digestion Methods.

8.3.2 Clean Up Optimization

Recoveries of clean up procedures were compared and results are presented in Figure 8-3. In general, sample filtration was the only method that achieved a good recovery with all AEDs together in one step regardless of their chemical properties (> 50 %). Vigabatrin had a low recovery (15 %) which was due to the digestion procedure. This drug did not exhibit a higher recovery with any of the methods used earlier.

Furthermore, all drugs exhibited an acceptable recovery with LLE except the amphoteric drugs (GBP, PGR and VIG) and LEV which did not show any improvement by changing the LLE solvent. LLE with ethyl acetate on its own or with IPA had slightly higher recoveries compared with DCM except for TIG which exhibited a better recovery with

DCM (> 40%) compared to ethyl acetate (< 5%). p-HPPH gave a poor recovery with DCM extraction (< 7%).

Consequently, methanolic extraction (neutral, basic and acidic) was chosen for further investigation and sample filtration was considered the clean-up procedure of choice in this study as a simple clean-up procedure to maintain optimal system performance.

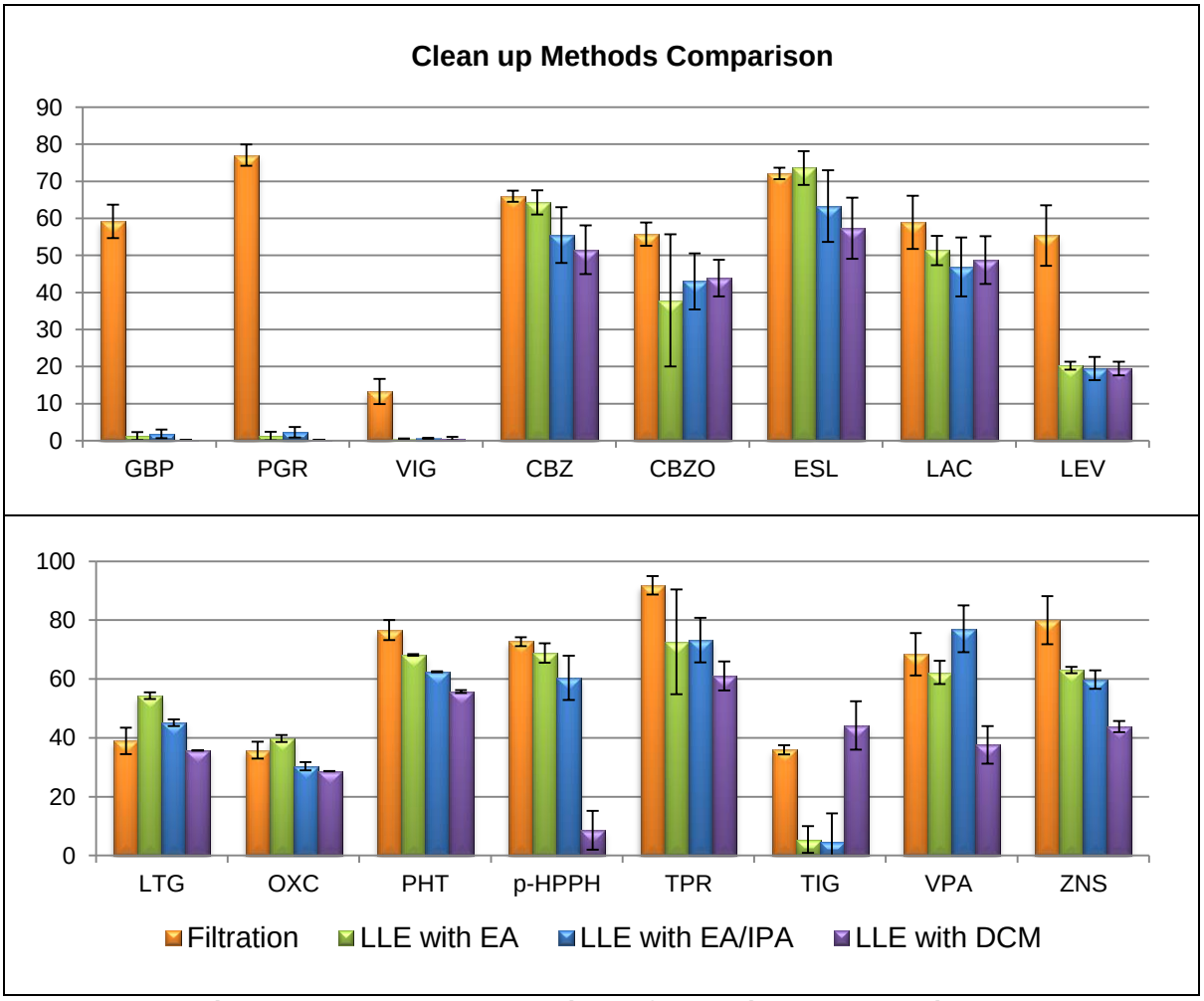


Figure 8-3: Recovery Comparison of Four Clean Up Procedures.

8.3.3 Investigation Into Extraction Conditions

8.3.3.1 Incubation Duration and Temperature

Recoveries of four digestion conditions were compared and results are presented in Figure 8-4. For all digestion methods, the highest recoveries for AEDs were attained with samples incubated for 1 hour at 60ºC and left overnight, with the exception of VIG and VPA. VIG and VPA exhibited higher recoveries when samples were incubated for only 2 hours at 60ºC. On the other hand, samples incubated at 40ºC for 2 hours or for 1 hour at 4ºC and left overnight exhibited the lowest recoveries with all drugs. Digestion at 60 ºC for

2 hours only or for 1 hour and leaving samples overnight gave similar recoveries for most of the drugs, except for OXC when extracted using acidic conditions. In general, increasing both temperature and incubation period improved the extraction recovery. Hence, digestion temperature and incubation duration could affect the extraction yield.

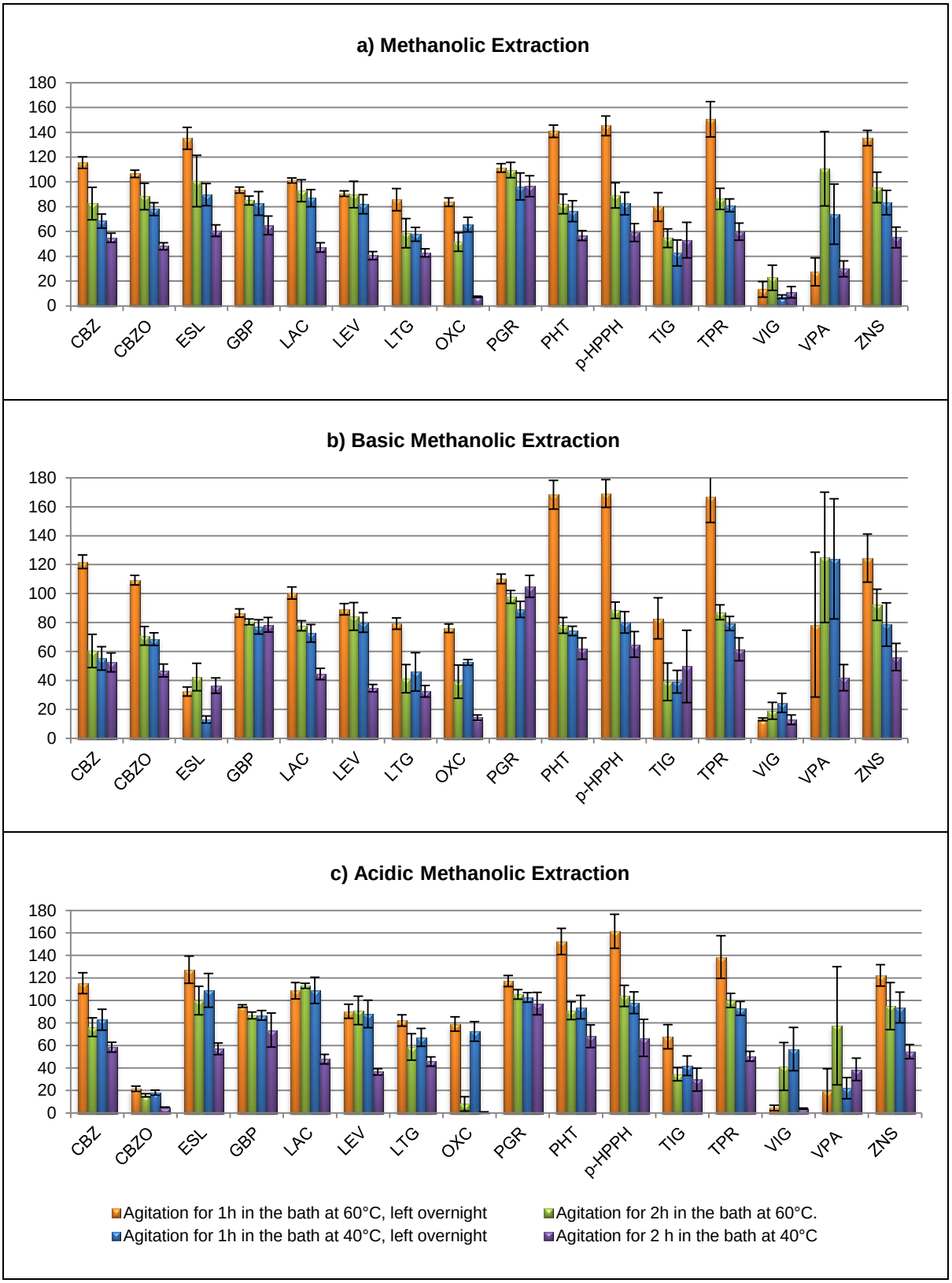


Figure 8-4: Effect of Incubation Duration and Temperature Condition on AED Recovery in Hair.

8.3.3.2 Sample Filtration Effect on Recovery

From Figure 8-5, it can be seen that the filtration step reduced AED recoveries by 10 - 30%. However, recovery values were still acceptable for filtered extracts. Furthermore, the use of filtration did not affect the drug chromatogram and decreased the baseline noise. Despite the slight decrease in recovery, the filters were continued to be used during method validation and hair sample analysis due to the resulting dirty hair extracts following digestion. Hence, a clean up procedure would help to maintain the instrument performance and decrease its down time due to residue build up and blockage of the LC system and decrease the matrix effect that would affect the mass spectrometry performance.

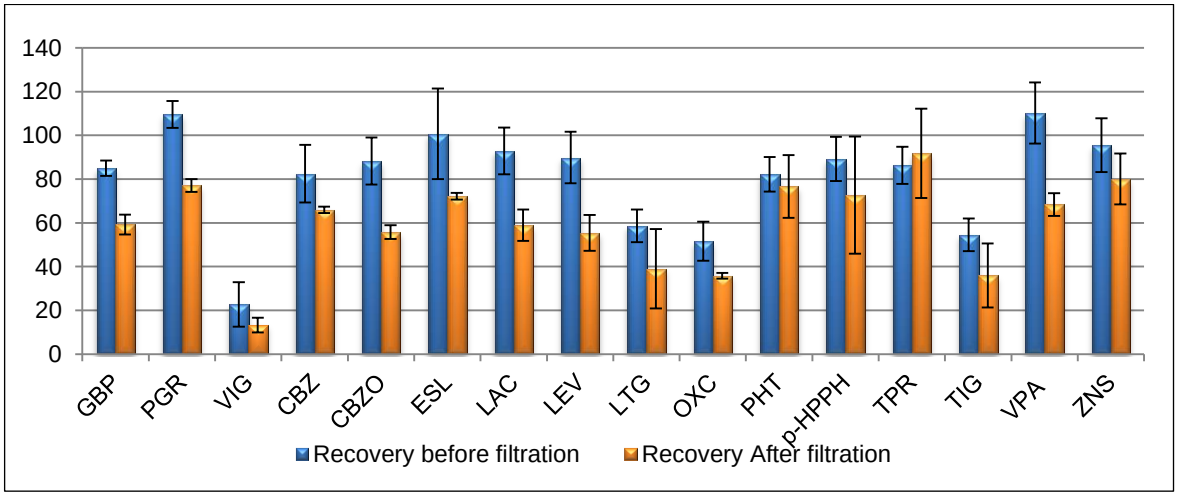


Figure 8-5: Sample Filtration Effect on Recovery of 16 AEDs.

8.3.4 Qualitative Method Validation

8.3.4.1 Selectivity

No endogenous or exogenous interferences were observed and none of the AEDs or their internal standards showed any interference at the retention time of the other drugs included in the method as illustrated in Figure 8-6 (a-e).

(a)

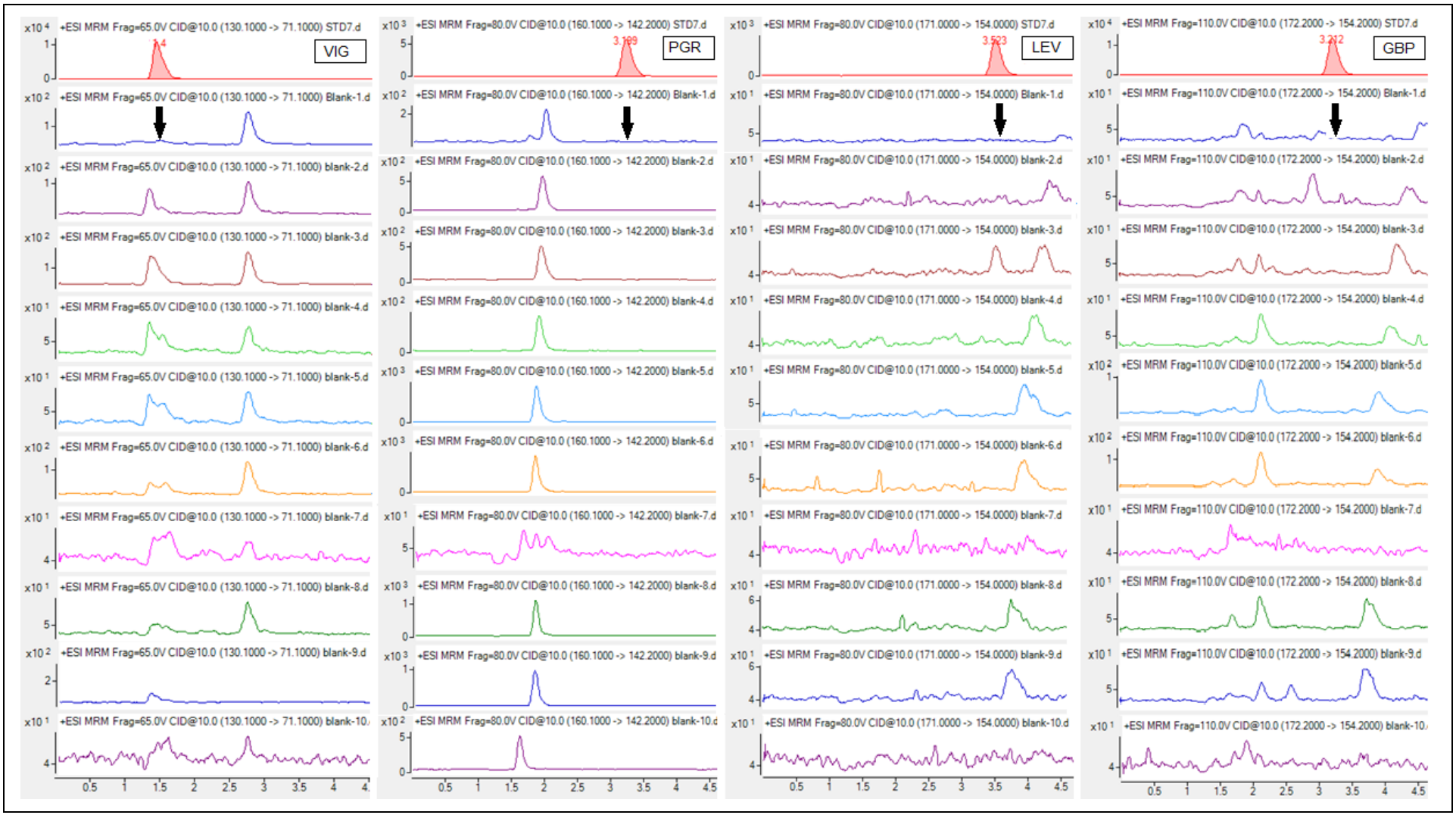


Figure 8-6 (a-e): The Chromatograms of 10 Drug-Free Hair Samples Compared to AED Mix Spiked Standard at Concentration of 2 ng/mg.

(b)

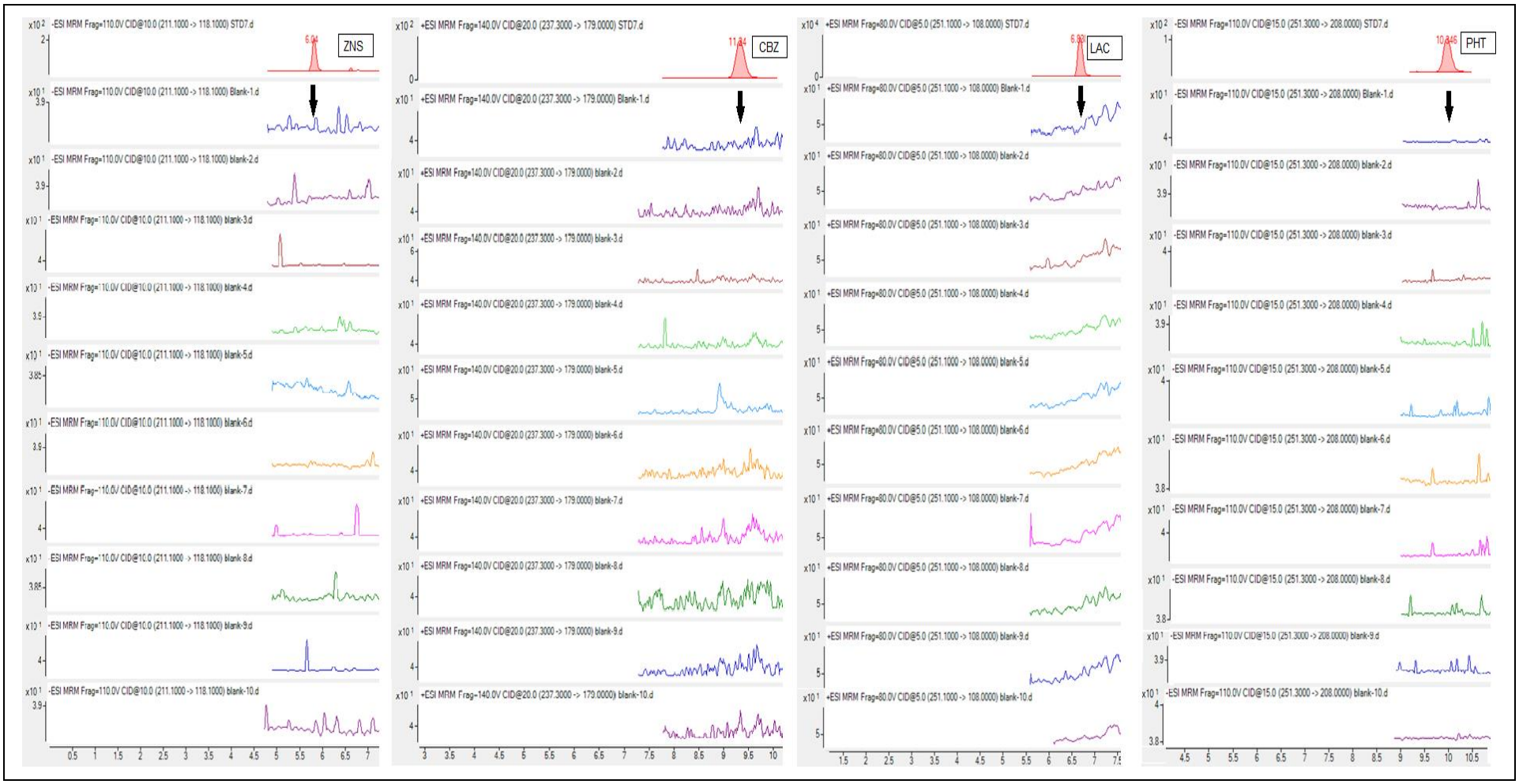


Figure 8-6 (a-e): The Chromatograms of 10 Drug-Free Hair Samples Compared to AED Mix Spiked Standard at Concentration of 2 ng/mg (Continued…).

(c)

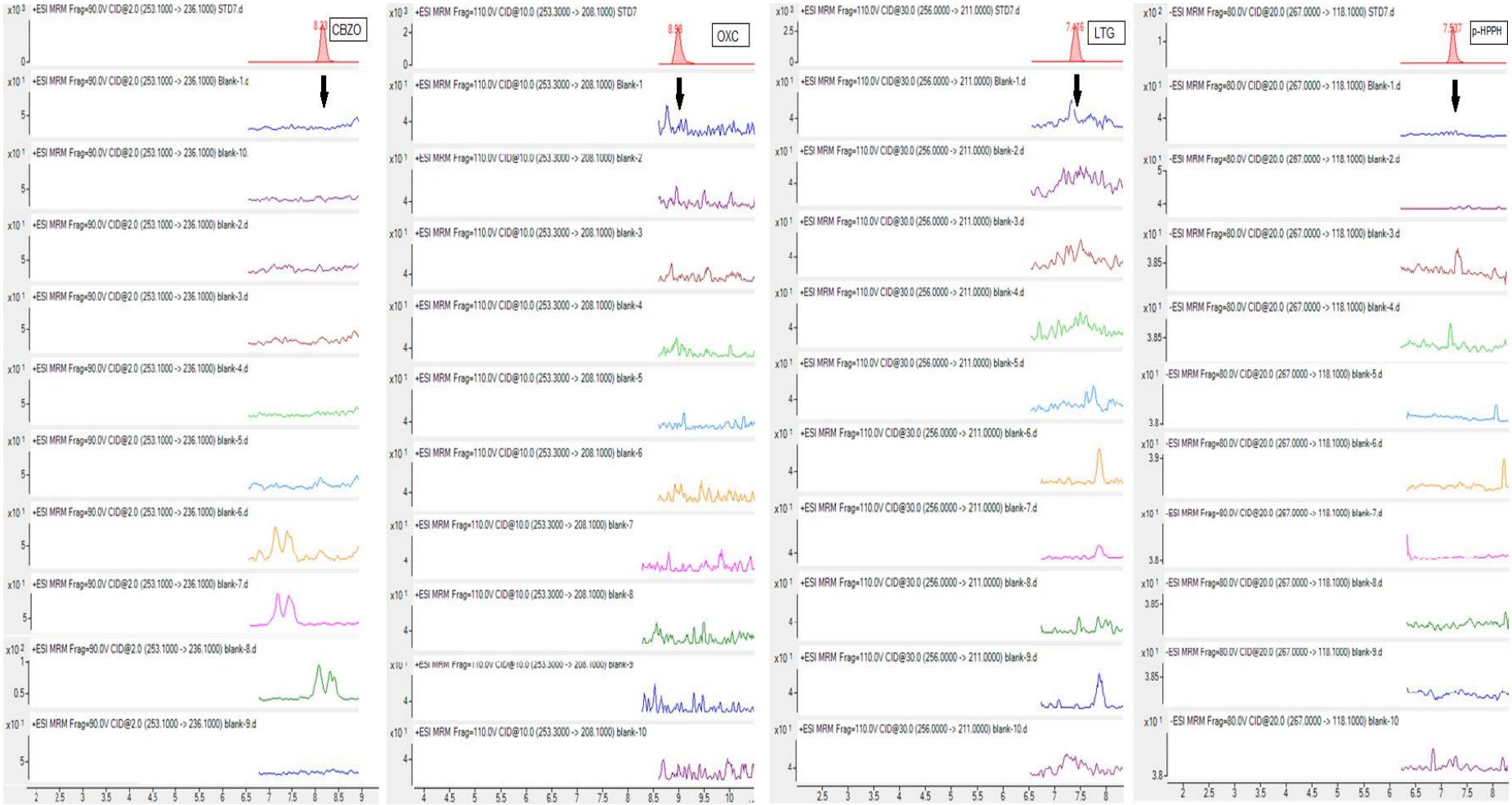


Figure 8-6 (a-e): The Chromatograms of 10 Drug-Free Hair Samples Compared to AED Mix Spiked Standard at Concentration of 2 ng/mg (Continued…).

(d)

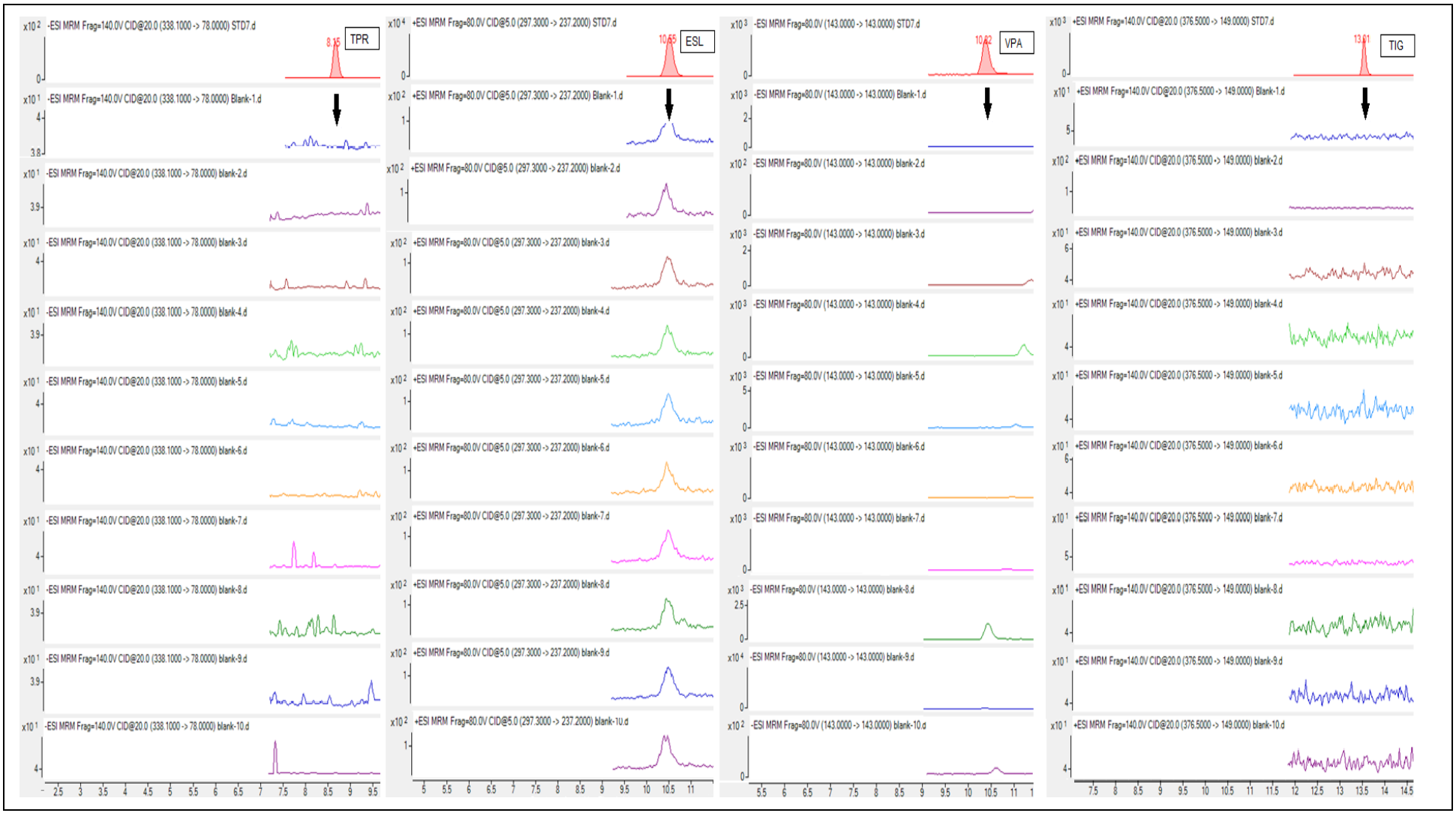


Figure 8-6 (a-e): The Chromatograms of 10 Drug-Free Hair Samples Compared to AED Mix Spiked Standard at Concentration of 2 ng/mg (Continued…).

(e)

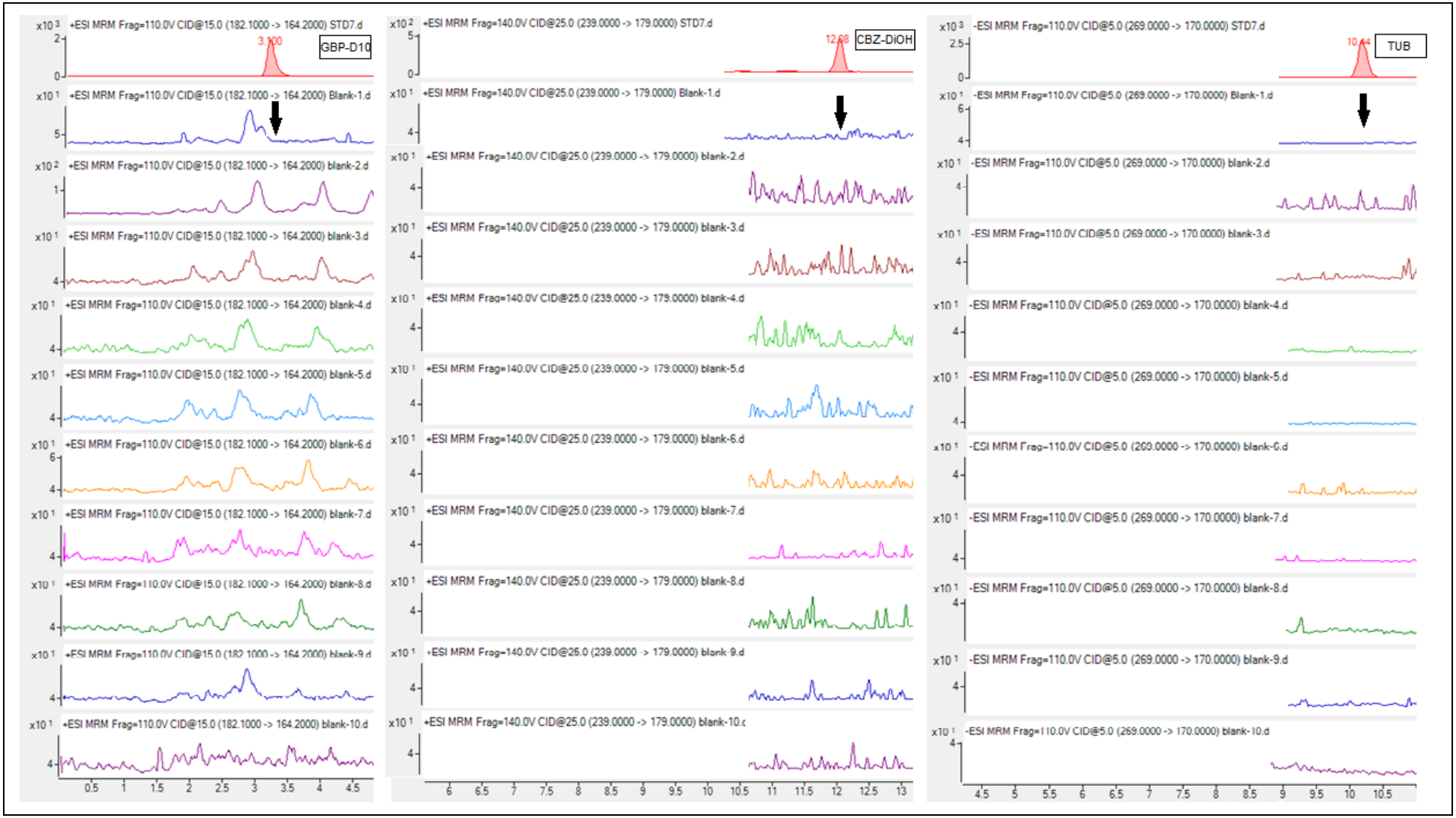


Figure 8-6 (a-e): The Chromatograms of 10 Drug-Free Hair Samples Compared to AED Mix Spiked Standard at Concentration of 2 ng/mg (Continued…).

8.3.4.2 Limit of Detection

LOD, LLOQ and LOQ results are presented in Table 8-4.

Table 8-4: LOD, LLOQ and LOQ of 16 AEDs in Hair.

AEDs	LOD (ng/mg)	LLOQ (ng/mg)	LOQ (ng/mg)
CBZ	0.025	0.05	0.05
CBZO	0.025	0.05	0.05
ESL	0.025	0.05	n/a
GBP	0.025	0.05	0.05
LAC	0.025	0.05	n/a
LEV	0.025	0.05	n/a
LTG	0.05	0.1	0.1
OXC	0.025	0.05	n/a
PGR	0.025	0.05	n/a
PHT	0.1	0.2	n/a
p-HPPH	0.1	0.2	n/a
TIG	0.1	0.2	n/a
TPR	0.025	0.05	n/a
VIG	0.05	0.1	n/a
VPA	0.01	0.05	0.05
ZNS	0.1	0.2	n/a

8.3.4.3 Recovery and Matrix Effect

The matrix factor and recovery results of 2 QCs (low and high) using 10 different sources of hair are detailed in Table 8-5. Recoveries were higher than 72.9 % for all AEDs except valproic acid which had a recovery of 50.8 % at the low concentration. Despite the high recoveries, the matrix factors exhibited low values outside the acceptable range (0.75-1.25) for most of the AEDs at the low concentration, except with ESL, LEV, PHT, p-HPPH and TPR. Ionization suppression occurred with all AEDs (<1) except OXC and VPA which exhibited an ionization enhancement effect (>1). One of the reasons for MF variation may be due to the way samples were prepared. Pre and post extracted samples were filtered whereas the non-extracted standards were injected directly. It was shown before that the filtration step reduced the recovery and since the MF calculated using post extracted samples divided by non-extracted samples, the variation may be due to drug loss during the filtration step rather than a matrix suppression effect. This explanation is supported by the high recovery values obtained when pre and post extracted samples were used for the recovery calculation and these were both filtered before extraction. Usually when matrix suppression is presented, it is associated with low extraction yield (see 2.3.8.4).

Table 8-5: Recovery and Matrix Factor Values for 16 AEDs Using Low and High QCs and 10 Different Hair Sources *(n=6 per QC per Matrix).*

AEDs	QC1		QC2	
	Recovery(%)	*Matrix Factor*	*Recovery(%)*	*Matrix Factor*
CBZ	87.1 ± 8.4	0.7 ± 0.1	78.2 ± 8.3	0.6 ± 0.1
CBZO	87.5 ± 6.4	0.7 ± 0.1	81.8 ± 8.2	0.6 ± 0.1
ESL	85.3 ± 9.6	0.8 ± 0.1	77.9 ± 8.6	0.7 ± 0.1
GBP	87.4 ± 11.0	0.5 ± 0.1	80.1 ± 9.1	0.5 ± 0.1
LAC	95.1 ± 16.7	0.5 ± 0.1	80.9 ± 12.6	0.5 ± 0.1
LEV	92.3 ± 9.8	0.8 ± 0.1	88.5 ± 19.7	0.7 ± 0.1
LTG	72.9 ± 19.0	0.4 ± 0.1	75.5 ± 8.9	0.5 ± 0.1
OXC	82.4 ± 7.5	1.7 ± 0.6	79.7 ± 8.9	1.6 ± 0.6
PGR	90.0 ± 11.1	0.6 ± 0.1	80.4 ± 10.3	0.6 ± 0.1
PHT	76.0 ± 5.1	0.8 ± 0.1	91.1 ± 34.3	0.6 ± 0.2
p-HPPH	86.0 ± 11.5	0.9 ± 0.2	81.2 ± 10.1	0.6 ± 0.1
TIG	104.3 ± 49.8	0.2 ± 0.1	71.4 ± 23.7	0.3 ± 0.1
TPR	82.7 ±11.5	0.9 ± 0.2	81.2 ± 10.1	0.6 ± 0.1
VIG	87.0 ± 7.0	0.3 ± 0.1	77.9 ± 12.4	0.3 ± 0.1
VPA	50.8 ± 16.7	1.2 ± 0.3	75.9 ± 9.7	1.5 ± 0.9
ZNS	76.0 ± 9.7	0.9 ± 0.2	82.5 ± 7.0	0.7 ± 0.1

8.3.4.4 Carryover

No carry over was observed for all 16 AEDs in hair. The percentage of carryover after the first blank injection was 0% for all drugs except carbmazepine epoxide, eslicarbazepine acetate, gabapentin, oxcarbazepine and vigabatrin which was 0.21%, 0.79%, 0.18%, 0.08% and 0.31% respectively. However, these percentages are acceptable (<10%) and very low compared to the high concentrations used (2 ng/mg). A summary of the carryover results is detailed in Table 8-6.

Table 8-6: Carryover Results of 16 AEDs After Double Injections of ULOQ (2 ng/mg) in Hair.

AED	Peak Area				Carryover (%)
	QC3 (n=2)	Blank 1	Blank 2	Blank 3	
CBZ	413565	0	0	0	**0.00**
CBZO	39204	84	33	0	**0.21**
ESL	336109	2671	2094	0	**0.79**
GBP	70966	124	62	0	**0.18**
LAC	131218	0	0	0	**0.00**
LEV	28589	0	0	0	**0.00**
LTG	10269	0	0	0	**0.00**
OXC	21483	16	0	0	**0.08**
PGR	36642	112	125	0	**0.00**
PHT	559	0	0	0	**0.00**
HPPH	497	0	0	0	**0.00**
TIG	6870	0	0	0	**0.00**
TPR	5231	0	0	0	**0.00**
VIG	17414	53	86	0	**0.31**
VPA	8960	0	0	0	**0.00**
ZNS	952	0	0	0	**0.00**

8.3.5 Case Samples

Carbamazepine, gabapentin and lamotrigine were detected in sample 1 and valproic acid was detected in sample 2 as expected. Chromatograms of AEDs detected in both samples are presented in Figure 8-7.

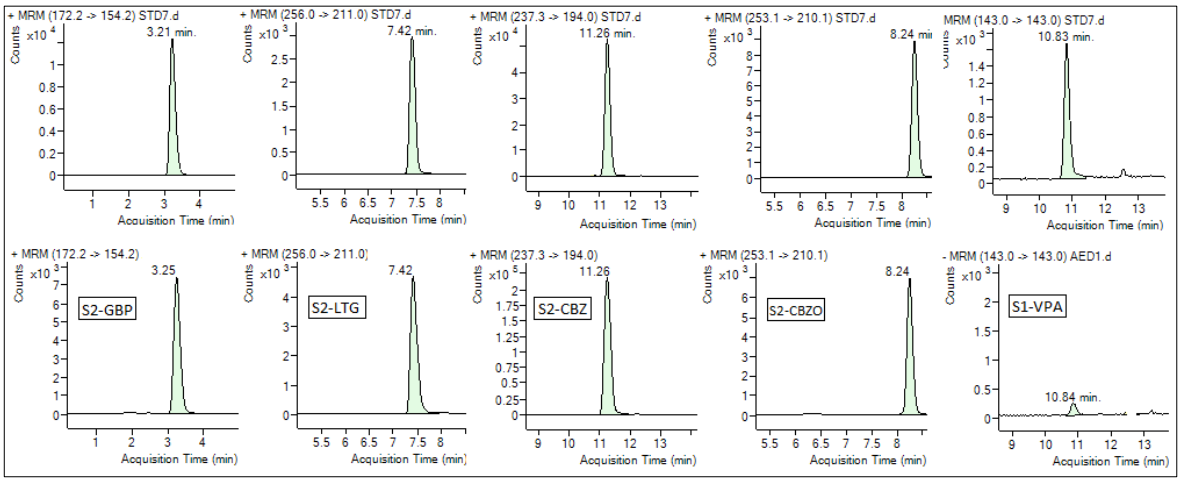


Figure 8-7: Chromatograms of AEDs Detected in Both Samples Compared to Non-Extracted Standard at 1 ng/mg.

In order to quantitate the AED concentrations, linearity, precision and accuracy checks were carried out. The calibration curves were linear with a R^2 greater than 0.998. Accuracy and precision results are presented in Table 8-7. Accuracy was within 18% of the nominal concentration and ranged between 82.6 to 115.8%. Precision was less than 15% for all AEDs except for the LTG low QC which had a precision of 17.5%.

Table 8-7: Accuracy and Precision Results of 4 AEDs and 1 Metabolite in Hair.

AEDs	Precision% (n=6)		Accuracy% (n=6)	
	0.5 ng/mg	**1 ng/mg**	**0.5 ng/mg**	**2 ng/mg**
CBZ	9.3	9.9	105.1	82.6
CBZO	15.8	5.5	109.6	106.5
GBP	6	2	105.5	106.4
LTG	17.5	13.6	103.8	87.6
VPA	4.9	14.2	115.8	82.7

AED samples were extracted using the two different digestion conditions that gave the highest recoveries with spiked hair samples in order to confirm the results obtained previously. The two conditions chosen were digestion for 2 hours only followed by direct filtration and for 1 hour and left overnight before filtration. Both digestions were at 60 ºC. Samples were digested and analysed in triplicate as described in 8.2.5.1.

AED calculated concentrations are presented in Figure 8-8. The results were comparable using both spiked and real hair samples. Carbamazepine, carbamazepine epoxide and gabapentin concentrations increased from 11.3, 4.0 and 2.0 ng/mg to 12.4, 6.2 and 2.9 ng/mg respectively when samples were left over night. Lamotrigine concentrations did not show any variation with a concentration of approximately 2.8 ng/mg. Finally, valproic acid has a higher recovery when samples were filtered and injected directly after 2 hours digestion (0.77 ng/mg) compared to samples left overnight (0.37 ng/mg).

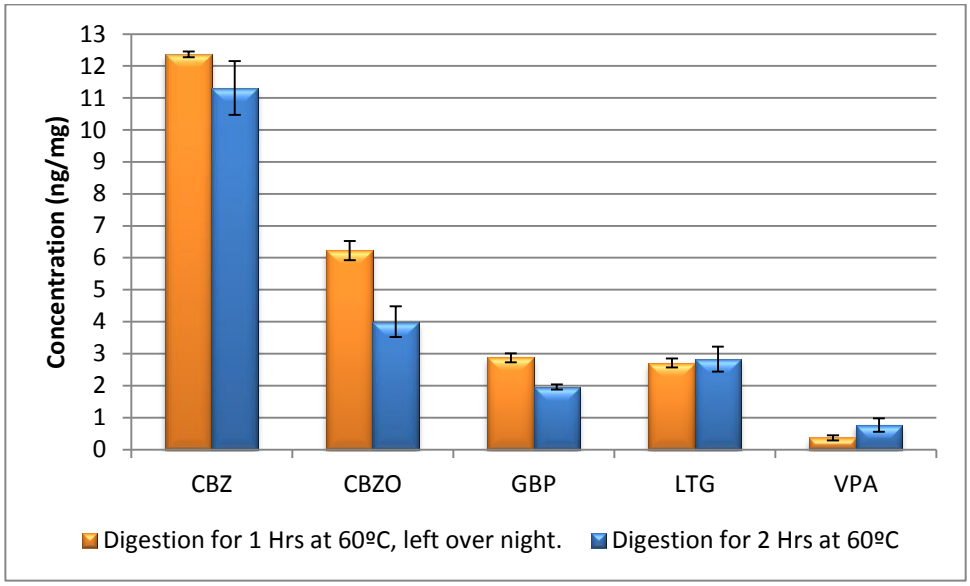


Figure 8-8: Mean Concentrations of 4 AEDs Detected in 2 Hair Samples Using two Digestions Conditions.

8.4 Conclusion

This is the first study to investigate the extraction optimization of 16 AEDs in hair of which 6 had been tested in hair previously; only carbamazepine, valproic acid, phenytoin, lamotrigine, oxcarbazepine and pregabalin were reported previously in the literature.

Six different digestion procedures using different neutral, basic and acidic solvents were compared. Methanolic digestion gave acceptable results with all AEDs including amphoteric drugs; gabapentin, pregabalin and vigabatrin. Amphoteric drug recovery was very low with strong acidic and basic digestion followed by liquid-liquid extraction as a cleaning-up procedure which may be due to the LLE step. Their recovery did not improve when LLE solvents were changed from DCM to ethyl acetate and IPA. Hence, sample filtration was considered the method of choice. Although the filtration step causes a 10-30% decrease in recovery, a sample cleaning-up procedure was an essential step to maintain good analysis and instrument performance.

Digestion incubation and temperature also had a significant effect on the sample extraction yield. Increasing both of these resulted in increasing AED recovery; however, caution should be taken when extraction of thermally labile drugs is carried out. Real case samples were used to compare the digestion effect and duration on drug recovery and the results were comparable for those obtained using spiked hair samples.

Finally, the method was qualitatively validated and 2 case samples from known AED users were successfully analysed. The samples were positive for the prescribed AEDs; carbamazepine and its metabolite carbamazepine epoxide, gabapentin, lamotrigine and valproic acid. However, no dose information was provided, hence the data could not be compared to what has been published in the literature.

9 Conclusion and Future Work

9.1 Conclusion

Antiepileptic drugs and their potential role in forensic cases have been investigated in this research. A significant growth in their reported abuse was noticed since 2011. Their multiple mechanisms of action on the CNS extended their indications to include a variety of psychological disorders and alcohol and substance misuse programmes although their efficacy in most of these indications is still under investigation. Their off-label prescription played a key role in their abuse potential. As well as their abuse with other illicit drugs and alcohol to increase euphoric effects, these drugs are being misused as pain killers.

The prevalence and abuse potential of AEDs among prison populations were evaluated. The study showed a high prevalence of AEDs (18%) largely due to GBP (13%) and PGR (4%) on their own or in combination with other drugs of abuse. However, the lack of information provided along with each sample did not allow differentiating between prescribed and abused AEDs.

A simple and accurate analytical method was successfully developed and validated for the simultaneous analysis of commonly encountered AEDs in different biological matrices. AEDs were extracted from whole blood, plasma, serum, urine and hair using a simple and cheap extraction procedure and a small sample volume (20 mg for hair and 100 µL for the other matrices) and analysed using LC/MS/MS QQQ. A wide concentration range to include therapeutic and toxic concentrations was used.

Firstly, the method was developed and quantitatively validated for the simultaneous analysis of 15 AEDs and 2 major metabolites in post mortem whole blood and was to be suitable for routine clinical and forensic toxicological analysis. The complex mixture of AEDs used in this method required a general method in order to attain high recoveries with all drugs of interest. Strata X cartridges are recommended for general SPE extraction; however, they did not achieve the optimum results for AEDs. Even though protein precipitation does not produce a particularly clean extract, the purity of the samples was sufficient to be injected into LC/MS/MS when a large reconstitution volume was used (1.5 mL). In addition, it is simple, fast, time saving and little solvent is required. Furthermore, increasing the centrifuge speed and duration improved the recovery. It is important also as

part of any method development to evaluate the effect of reconstitution volume and composition due to its significant effect on linearity, LOQ and calibration model.

Despite the complex composition of postmortem blood samples which lead to the use of a large sample volume in order to have a homogenous sample, the method was successfully validated using a small sample volume (100 µL).

Simultaneous methods can be a great advantage for routine laboratory work. Despite the high cost of this method at the development stage, on a long-term basis the method would save on analyst time, consumable cost and effort. Rather than using many different methods to test for these substances individually the same procedure can be used. It may also reveal the presence of other drugs that have not been mentioned in the medical history either prescribed or misused. Furthermore, simultaneous analysis presents an important tool for TDM where AEDs are taken concomitantly and the concentration variations could significantly affect the quality of life for people with epilepsy related conditions.

The method was transferred to another laboratory and extended to include 22 AEDs. It was validated for serum and plasma in addition to whole blood. The main factors required to be taken into consideration when transferring the LC/MS/MS method from one instrument to another or from one laboratory to another were assessed as well. In order to transfer an LC/MS/MS method, collision energy and fragmentor voltage have to be assessed to attain the highest sensitivity. Mobile phase and column conditions can be easily transferred.

The first stability study to specifically investigate the stability of 16 AEDs in postmortem blood was carried out. Drug stability is very important in forensic cases where analysis may be delayed due to the time needed for the autopsy, chain of custody, sending samples for analysis and generating results and sometimes sample reanalysis if requested by court or police. During this long process, samples may be exposed to various storage conditions which can affect the concentration of drugs and other chemicals and lead to false negative or positive results. Hence, well designed stability studies under different conditions are required to interpret the toxicological findings correctly. Since some drugs are more sensitive to storage conditions, it is important during any stability study to report the temperature at which the study was carried out especially at room temperature. It was noticed that the stability of some drugs significantly decreased when room temperature increased from 18°C to 25°C (retigabine, eslicarbazepine acetate and oxcarbazepine).

Finally, hair as a biological matrix represents a valuable alternative when other biological samples are not available or when a history of drug use is required. The extraction of 16 AEDs in hair was optimized. Methanolic extraction was the method of choice for all AEDs including amphoteric drugs. Amphoteric drug recovery was very low with all strong acidic and basic digestions followed by liquid-liquid extraction as cleaning-up procedure. Digestion incubation and temperature as well has a significant effect on sample extraction yield. Increasing both of these resulted in enhancing AEDs recovery; however, caution should be taken into account when extraction of thermally labile drugs is carried out. Finally, the method was qualitatively validated and 2 case samples from known AED users were successfully analysed.

9.1 Future Work

Awareness regarding these drugs and their probability to be abused among GPs and other health organisations should be raised. Some guidance for prescribing should be established and more care should be considered when they are prescribed especially for people with pervious abuse history.

A larger scale study to include addiction and pain management clinics in addition to prisons is recommended to assess AEDs abuse prevalence among substance abusers and pain killer misusers. It would be an advantage to obtain more accurate data regarding sex, age and medical history to generate more accurate statistics.

It was noticeable in this research that AED concentrations, especially gabapentin and pregabalin, in some samples were above the reported therapeutic limits in the literature. Hence, a study to establish the toxic limits of gabapentin and pregabalin concentrations in blood is recommended which would aid the pathologist in the interpretation of results in postmortem cases. Furthermore, a study is required to compare AEDs concentrations in postmortem SUDEP cases in order to investigate any relationship between the number and/or concentrations of AEDs taken at the time of death and SUDEP as a cause of death.

Simultaneous analysis for multiple drugs of abuse using LC/MS/MS would be a great advantage in forensic toxicology where most postmortem cases usually include many drugs being abused together and not all being declared or requested for analysis.

Stability of retigabine and its metabolite requires further investigation to include solvents, blood stabilizers, and storage condition in order to ensure accurate quantitive analysis that reflect the actual concentration in biological fluids.

Finally, AEDs analysis in hair would require an extended validation with a larger number of authentic samples to include all drugs of interest in order to prove the extraction method efficiency and be applicable for forensic and therapeutic drug monitoring analysis.

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Appendices

Appendix 1-1: An Overview of Data From Reports of AED Abuse.

RF	AED	Subject (y/g)	Case	Dose/day	Plasma Conc. (mg/L)	Symptoms	Drug source	RA
(37), 1996	GBP VPA	32/M	GBP, VPA and alcohol misuse	91 g GBP 54 g VPA	44.5 GBP 107.1 VPA	Dizziness, sleepiness, depressed, slurred speech, suicidal attempt	Prescription for treating epilepsy	Oral
(94), 1997	GBP	41/F	GBP dependence	600-1500 mg	N/A	"Laid-back" feelings, decreased cocaine craving WS: Suicidal thoughts, depression	Her husband's gabapentin to wean her off Crack cocaine	Oral
(99), 2000	GBP	38/F	GBP prescription	900 mg	N/A	Euphoria, feeling well, inappropriate laughter, increased energy	Prescription for treating epilepsy and pain	Oral
(99), 2000	GBP	37/F	GBP prescription	1800 mg	N/A	Euphoria, feeling well, inappropriate laughter	Prescription for treating epilepsy	Oral
(95), 2007	GBP	67/F	GBP abuse WS	4200-7200 mg	N/A	WS: trembling, sweating, excitation, exophthalmia	Prescription for treating alcohol abuse	Oral
(98), 2007	GBP	33/M	GBP abuse WS	3600 mg	N/A	WS: tremulous, confusion, agitation, tachycardia, hyper-reflex, diaphoretic, disoriented	Prescription for cannabis and alcohol abuse treatment	Oral
(98), 2007	GBP	63/M	GBP abuse WS	4900 mg	N/A	Fatigue, increasing sedation, confusion	Prescription as analgesic	Oral
(122), 2009	GBP	44/M	Driving under GBP effects	N/A	<0.2	Unfocused, slow, aggressive	Self-medicating with GBP and quetiapine for bipolar	Oral
(122), 2009	GBP	24/F	Driving under GBP effects	300 mg	15.5	Impaired with thick, slurred speech, slow reactions, sleepiness	Prescription with quetiapine	Oral
(122), 2009	GBP	51/M	Driving under GBP effects	N/A	2.5	Slow, deliberate movements, lethargy	N/A	Oral

RF: reference, RA: Route of administration, F: female, M: male, N/A: information not applicable, PHT: phenytoin, VPA: sodium valproate, CBZ: carbamazepine, GBP: gabapentin, PGR: pregabalin, VIG: vigabatrin, TIG: tiagabine, ZNS: zonisamide, LAC: lacosamide, OXC: oxcarbazepine, TPR: topiramate, LTG: lamotrigine, BNZ: benzodiazepines, AW: alcohol withdrawal, WS: withdrawal symptoms.

Appendix 1-1: An Overview of Data From Reports of AED Abuse (continued…).

RF	AED	Subject (y/g)	Case	Dose/day	Plasma Conc. (mg/L)	Symptoms	Drug source	RA
(102), 2010	GBP	53/F	GBP withdrawal syndrome	700 mg	N/A	WS: confusion, agitation, anxiety, headache, nervousness.	Prescription as analgesic for liver cirrhosis, resulting from alcohol abuse	Oral
(122) 2009	GBP	24/M	Driving under GBP effects	N/A	4.4	Subdued, lethargy, scattered thoughts, confusion	Prescription for psychiatric disorder	Oral
(105), 2010	PGR	47/M	PGR abuse with alcohol and cannabis	7500 mg	29	Euphoria WS: sweating, unrest, hypertension, tremor, craving for PGR	A friend who was prescribed PGR for pain	Oral
(103), 2010	PGR	28/F	PGR and alcohol abuse	N/A	N/A	Psychotic reaction, became high	N/A	Oral
(103), 2010	PGR	18/M	PGR and alcohol abuse	875 mg	N/A	Became high but in high dose developed generalized seizures	N/A	Oral
(103), 2010	PGR	35/M	PGR and substance abuse	525 mg	N/A	Euphoria, described as “amphetamine trip”	Prescription for anxiety	Oral
(103), 2010	PGR	23/M	Non-specific substance abuse	300 mg	N/A	Became high	N/A	Oral
(103), 2010	PGR	26/M	PGR and substance abuse	2400 mg	N/A	Hospitalisation for detoxification	Prescription for anxiety	Oral
(103), 2010	PGR	26/F	PGR and substance abuse	1200 mg	N/A	Slow tapering of dose required	Prescription for anxiety	Oral

RF: reference, RA: Route of administration, F: female, M: male, N/A: information not applicable, PHT: phenytoin, VPA: sodium valproate, CBZ: carbamazepine, GBP: gabapentin, PGR: pregabalin, VIG: vigabatrin, TIG: tiagabine, ZNS: zonisamide, LAC: lacosamide, OXC: oxcarbazepine, TPR: topiramate, LTG: lamotrigine, BNZ: benzodiazepines, AW: alcohol withdrawal, WS: withdrawal symptoms.

Appendix 1-1: An Overview of Data From Reports of AED Abuse (continued…).

RF	AED	Subject (y/g)	Case	Dose/day	Plasma Conc. (mg/L)	Symptoms	Drug source	RA
(103), 2010	PGR	43/F	PGR and BNZ abuse	1000 mg	N/A	Euphoria, described as "amphetamine trip", hyperactivity, decreased consciousness	N/A	Oral
(103), 2010	PGR	38/M	PGR and substance abuse	3000 mg	N/A	Suicide thoughts when decreasing dose	Prescription for anxiety	Oral
(103), 2010	PGR	51/M	PGR and substance abuse	N/A	N/A	Amnesia, suicide thoughts	N/A	Oral
(103), 2010	PGR	29/M	PGR and substance abuse	N/A	N/A	N/A	N/A	Oral
(103), 2010	PGR	32/M	PGR and substance abuse	N/A	N/A	Multiple hospital admission for PGR and zolpidem abuse	Patient sold his PGR tablets	Injection
(103), 2010	PGR	M	PGR and substance abuse	1050 mg	N/A	Nice BNZ effects	Prescription for anxiety	Oral
(103), 2010	PGR	19/M	PGR and substance abuse	300 mg	N/A	Became high, effects similar to diazepam	Prescription for anxiety	Nasally
(103), 2010	PGR	29/M	PGR and substance abuse	N/A	N/A	Behavioural disturbance	Patient sold her tablets on black market	Oral
(112), 2001	TPR	30/F	Topiramate abuse	450 mg	N/A	Decreased cognition, dulled thinking, blunted mental reactions, blurred vision, paresthesias, sleepiness, gastrointestinal disturbances	Prescription for treating mania	Oral
(113), 2004	TPR	17/F	Topiramate abuse	800 mg	N/A	Incoherence, confusion, disorientation, speech impairments	From friends	Oral

RF: reference, RA: Route of administration, F: female, M: male, N/A: information not applicable, PHT: phenytoin, VPA: sodium valproate, CBZ: carbamazepine, GBP: gabapentin, PGR: pregabalin, VIG: vigabatrin, TIG: tiagabine, ZNS: zonisamide, LAC: lacosamide, OXC: oxcarbazepine, TPR: topiramate, LTG: lamotrigine, BNZ: benzodiazepines, AW: alcohol withdrawal, WS: withdrawal symptoms.

Appendix 1-1: An Overview of Data From Reports of AED Abuse (continued…).

RF	AED	Subject (y/g)	Case	Dose/day	Plasma Conc. (mg/L)	Symptoms	Drug source	RA
(92), 1993	CBZ	24/F	Beer and CBZ abuse	1000-1800 mg CBZ	16.5	Euphoric feeling, light-headedness, dizziness	Prescription for BNZ and alcohol abuse treatment (300 mg/day)	Oral
(103), 2010	PGR	42/F	PGR and substance abuse	4200 mg	N/A	Psychosis requiring hospitalization	N/A	Oral
(92), 1993	CBZ	38/M	Abuse	1200 mg	18.1	Euphoric feeling, light-headedness, dizziness, ataxia, diplopia. WS appeared after drug discontinuation	Alcohol dependence treatment	Oral
(93), 1997	CBZ	22/F	Alcohol and CBZ dependence	N/A	N/A	Temazepam and alcohol-like euphoria	Purchased from the street	Oral
(110), 1993	PHT	40/M	Crack cocaine abuse with PHT	60 capsules	52	Difficulty walking, slurred speech, tremor, double vision and fatigue	Friends-street practice	Smoked
(110), 1993	PHT	42/M	Crack cocaine abuse with PHT	N/A	22	Double vision, unsteady gait, prominent nystagmus, ataxia (the symptoms appeared within the therapeutic range).	Prescribed for him as an epileptic patient	Smoked
(110), 1993	PHT	34/M	Crack cocaine abuse with PHT	N/A	50	Nystagmus, ataxia, lethargy, slurred speech	Prescribed for him as an epileptic patient	Smoked
(110), 1993	PHT	36/M 48/M	Crack cocaine abuse with PHT	N/A	7-8	No symptoms found	Street practice	Smoked
(111),1995	PHT	36/M	Crack cocaine abuse with PHT	N/A	28	Unsteady gait, nausea,	From a friend with epilepsy	Smoked
(79), 2004	PHT	19/M	Alcohol, marijuana and alcohol abuse	N/A	45	Slurred speech, somnolence, agitation, combative, ataxia	From street	Smoked

RF: reference, RA: Route of administration, F: female, M: male, N/A: information not applicable, PHT: phenytoin, VPA: sodium valproate, CBZ: carbamazepine, GBP: gabapentin, PGR: pregabalin, VIG: vigabatrin, TIG: tiagabine, ZNS: zonisamide, LAC: lacosamide, OXC: oxcarbazepine, TPR: topiramate, LTG: lamotrigine, BNZ: benzodiazepines, AW: alcohol withdrawal, WS: withdrawal symptoms.

Appendix 2-2: Tuning Results of 15 AEDs and 5 Internal Standards Using the Optimizer Software.

Optimizer Report

Project Name: AEDs
Instrument Name: Instrument 1
Instrument Model: G6420A

Compound Name	Formula	Mass	Sample Position
GBP		171.24	Vial 1
GBP		171.24	Vial 1

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
172.2	70	154.2	12	266570
172.2	70	137.1	16	109301
172.2	70	55.1	28	89458
172.2	70	95.2	24	53157

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-neg-test.m	Negative	ESI

Compound Name	Formula	Mass	Sample Position
VIG		129	Vial 3

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
130	90	71.1	12	225357
130	90	113.1	4	189689
130	90	67.1	20	41382
130	90	43.2	24	40952

Compound Name	Formula	Mass	Sample Position
DZM-5		289.74	Vial 20

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
290.75	35	140.3	28	58
290.75	35	167.9	12	67

Compound Name	Formula	Mass	Sample Position
PGR		159.1	Vial 2

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
160.1	100	142.2	8	2806512
160.1	100	55.2	24	2043869
160.1	100	83.2	12	554464
160.1	100	97.2	12	649107

Optimizer Report

Compound Name	Formula	Mass	Sample Position
VPA		144	Vial 4

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
145	125			

Compound Name	Formula	Mass	Sample Position
CBZ		236.27	Vial 5

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
237.3	100	99.1	20	253

Compound Name	Formula	Mass	Sample Position
OXC		252.27	Vial 6

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
253.3	100	59.3	12	241

Compound Name	Formula	Mass	Sample Position
ESC		296.32	Vial 7

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
297.3	85	194.2	20	1236
297.3	85	237.2	4	1474
297.3	85	59.1	4	764
297.3	85	179.3	40	247

Compound Name	Formula	Mass	Sample Position
PHT		252.27	Vial 8

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
253.3	80	59.1	8	315

Compound Name	Formula	Mass	Sample Position
LTG		256.09	Vial 9

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
257.1	80	153.7	20	73

Compound Name	Formula	Mass	Sample Position
TPR		339.36	Vial 10

Optimizer Report

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
340.4	100	196	12	78

Compound Name	Formula	Mass	Sample Position
TIG		375.5	Vial 11

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
376.5	110	263	12	72

Compound Name	Formula	Mass	Sample Position
RTG		376.23	Vial 12

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
377.2	100	273	4	67

Compound Name	Formula	Mass	Sample Position
LAC		250.29	Vial 13

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
251.3	75	59.3	12	356

Compound Name	Formula	Mass	Sample Position
LEV		170	Vial 14

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
171	90	126.1	12	439569
171	90	154.1	0	233101
171	90	69.1	28	90365
171	90	41.2	40	74157

Compound Name	Formula	Mass	Sample Position
ZNS		212	Vial 15

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
213	80	133	8	134

Compound Name	Formula	Mass	Sample Position
TUB		270.35	Vial 16

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Optimizer Report

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
271.4	90	189.1	4	382

Compound Name	Formula	Mass	Sample Position
GBP-10		181	Vial 17

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
182	70	164.2	12	170328
182	70	147.2	16	72968
182	70	55.1	24	50941
182	70	119.2	20	12848

Compound Name	Formula	Mass	Sample Position
PGR-6		165.2	Vial 18

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
166.2	145	148.2	8	15436
166.2	145	130.2	12	3305
166.2	145	103.3	12	2939
166.2	145	58.2	24	3689

Compound Name	Formula	Mass	Sample Position
TPR-12		351.36	Vial 19

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-pos-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
352.4	100	270.2	0	557
352.4	100	189.3	16	59
352.4	100	187	8	180
352.4	100	201.8	0	44

Optimizer Report

Project Name: AEDs
Instrument Name: Instrument 1
Instrument Model: G6420A

Compound Name	Formula	Mass	Sample Position
GBP		171.24	Vial 1

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
172.25	80	154.2	8	294510
172.25	80	137.1	16	130412
172.25	80	55.1	20	94817
172.25	80	95.2	20	54046

Compound Name	Formula	Mass	Sample Position
PGR		159.1	Vial 2

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
160.11	80	142.2	8	2044777
160.11	80	55.2	20	1328536
160.11	80	83.2	12	391155
160.11	80	97.2	12	451967

Optimizer Report

Project Name: AEDs
Instrument Name: Instrument 1
Instrument Model: G6420A

Compound Name	Formula	Mass	Sample Position
VIG		129	Vial 3

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-test.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
130.01	70	71.1	12	313873
130.01	70	113.1	4	261667
130.01	70	67.1	20	56397
130.01	70	43.2	20	55056

Optimizer Report

Project Name: AEDs
Instrument Name: Instrument 1
Instrument Model: G6420A

Compound Name	Formula	Mass	Sample Position
VPA		144	Vial 4

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-neg-test.m	Negative	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
145	100			

Compound Name	Formula	Mass	Sample Position
LTG		256.09	Vial 9

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-neg-test.m	Negative	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
257.1	90	153.7	16	40

Compound Name	Formula	Mass	Sample Position
TPR		339.36	Vial 10

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-neg-test.m	Negative	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
340.4	115	196	20	42

Compound Name	Formula	Mass	Sample Position
TUB		270.35	Vial 16

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-neg-test.m	Negative	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
271.4	75	189.1	0	44

Compound Name	Formula	Mass	Sample Position
TPR-12		351.36	Vial 19

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-neg-test.m	Negative	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
352.4	80	270.2	8	40
352.4	80	189.3	20	40
352.4	80	187	0	39
352.4	80	201.8	4	40

Compound Name	Formula	Mass	Sample Position
DZM-5		289.74	Vial 20

Method Name	Polarity	Ion Source
D:\Shaza\Methods\AEDs-neg-test.m	Negative	ESI

Optimizer Report

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
290.8	35	140.3	24	39
290.8	35	167.9	4	38

Optimizer Report

Project Name:
Instrument Name: Instrument 1
Instrument Model: G6420A

Compound Name	Formula	Mass	Sample Position
m-PHT		268.27	Vial 22

Method Name	Polarity	Ion Source
D:\MassHunter\methods\AEDs general.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
269.28	45	105	8	2184
269.28	45	187	4	1758

Compound Name	Formula	Mass	Sample Position
CBZ-O		252.27	Vial 23
CBZ-O		252.27	Vial 23

Method Name	Polarity	Ion Source
D:\MassHunter\methods\AEDs general.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
253.28	45	59.2	8	201

Method Name	Polarity	Ion Source
D:\MassHunter\methods\AEDs general--.m	Negative	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
251.26	30	251.9	12	105
251.26	30	152	0	116

Compound Name	Formula	Mass	Sample Position
CBZ-OH		238.28	Vial 24
CBZ-OH		238.28	Vial 24

Method Name	Polarity	Ion Source
D:\MassHunter\methods\AEDs general.m	Positive	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
239.29	40			

Method Name	Polarity	Ion Source
D:\MassHunter\methods\AEDs general--.m	Negative	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
237.27	45	59	12	108
237.27	45	158.9	36	96
237.27	45	59.5	32	107

Optimizer Report

Project Name:
Instrument Name: Instrument 1
Instrument Model: G6420A

Compound Name	Formula	Mass	Sample Position
m-PHT		268.27	Vial 22

Method Name	Polarity	Ion Source
D:\MassHunter\methods\AEDs general--.m	Negative	ESI

Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
267.26	35			

Optimizer Report

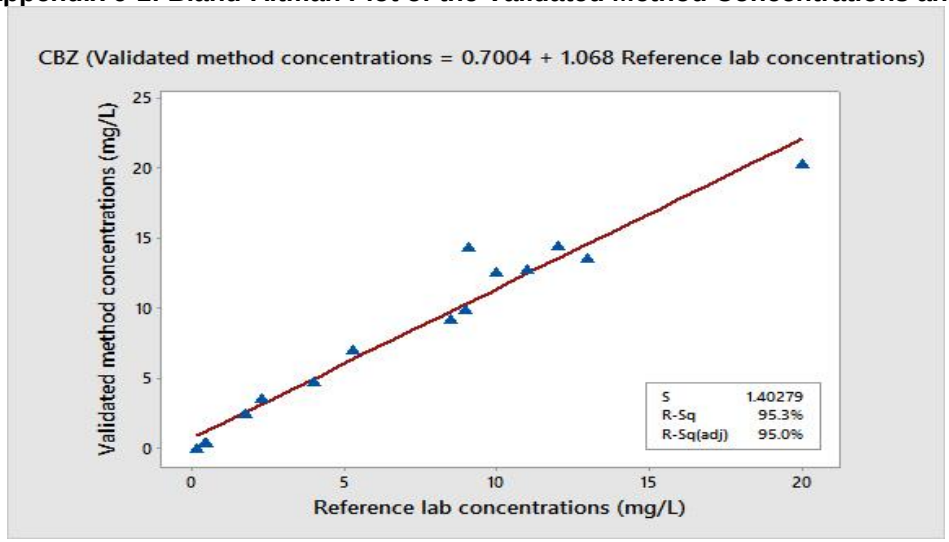
Project Name: AEDs
Instrument Name: Instrument 1
Instrument Model: G6420A

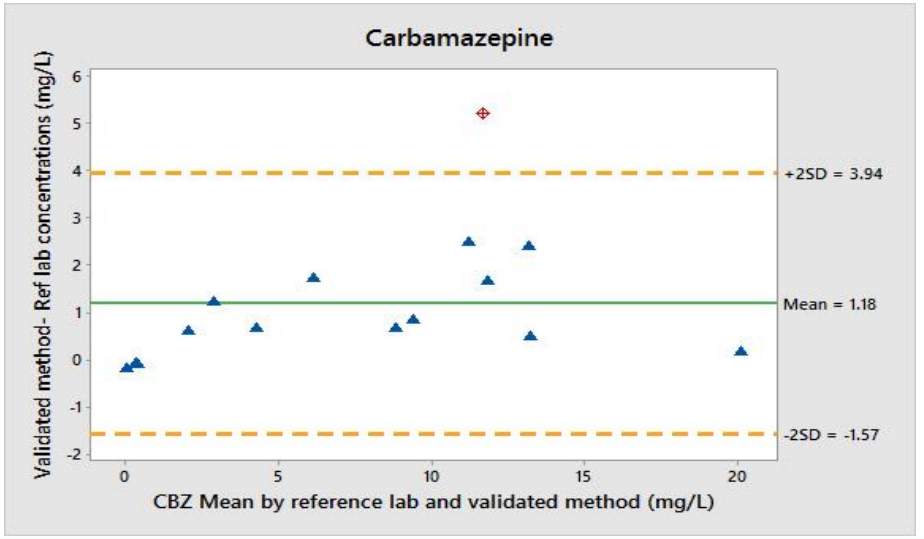
Compound Name	Formula	Mass	Sample Position
RTG		376.23	Vial 12

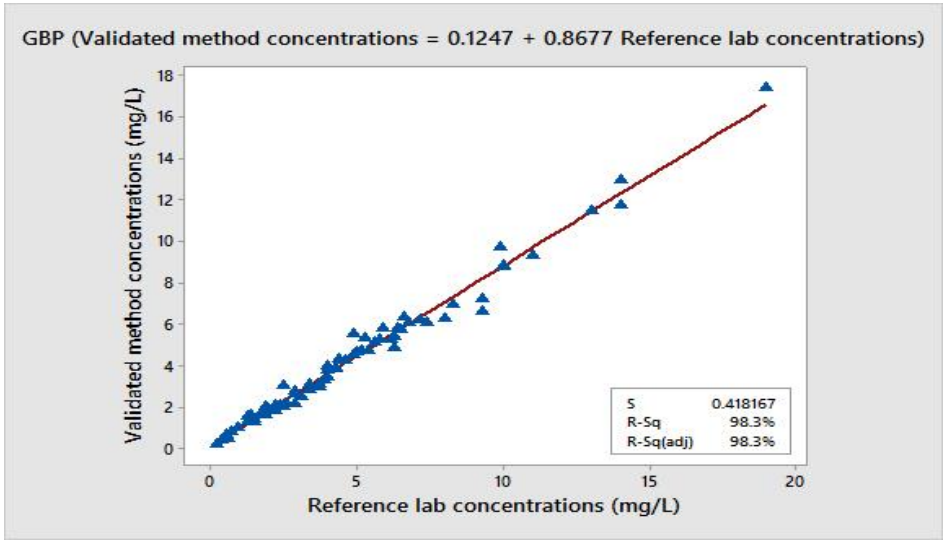
Method Name	Polarity	Ion Source
D:\MassHunter\methods\RTG.m	Positive	ESI

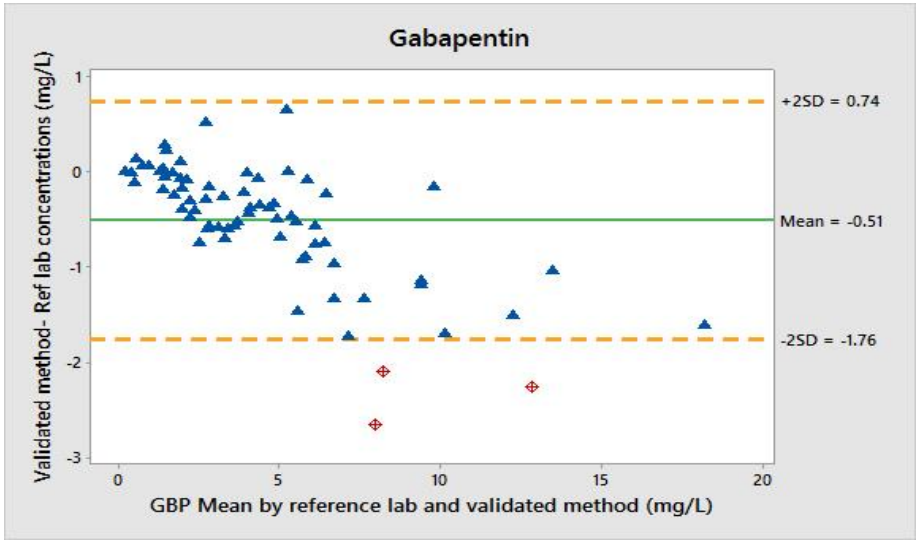
Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
377.24	45	214	8	115

Appendix 6-1: Bland-Altman Plot of the Validated Method Concentrations and the Reference Lab Concentrations for Each AED.

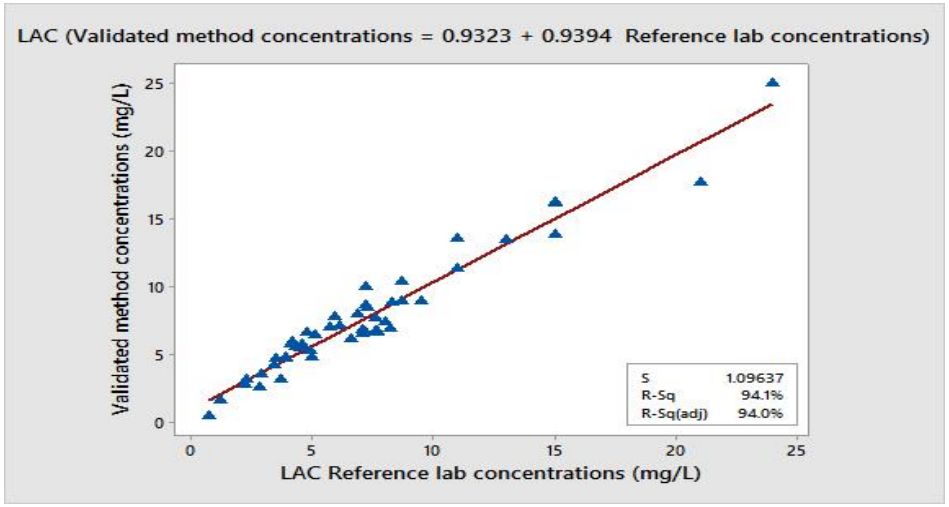


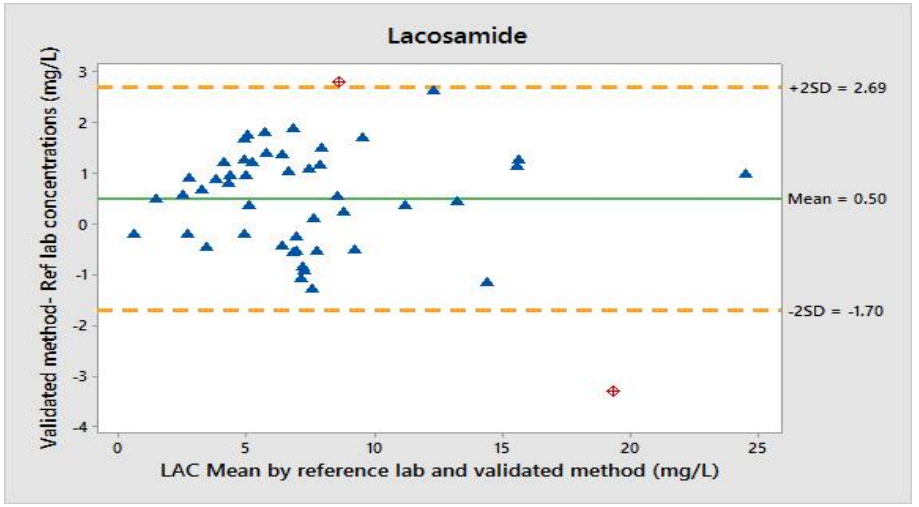


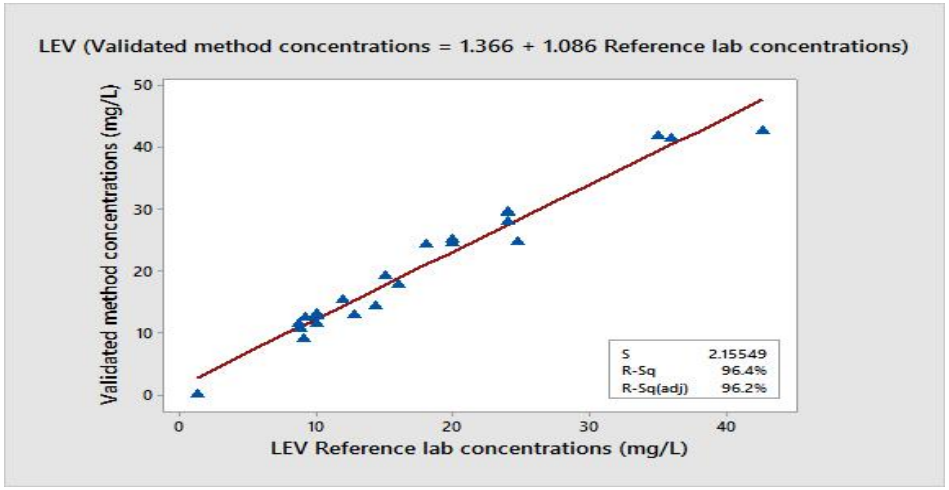


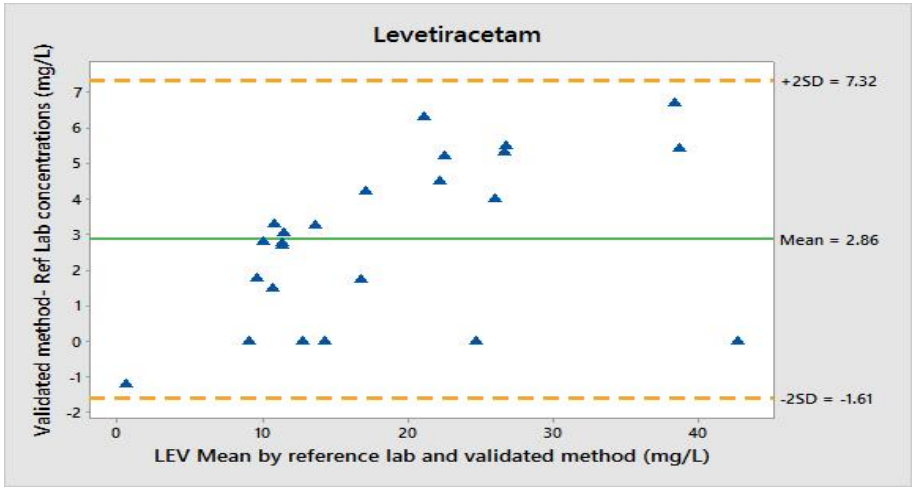


Appendix 6-1: Bland-Altman Plot of the Validated Method Concentrations and the Reference Lab Concentrations for Each AED (Continued…).

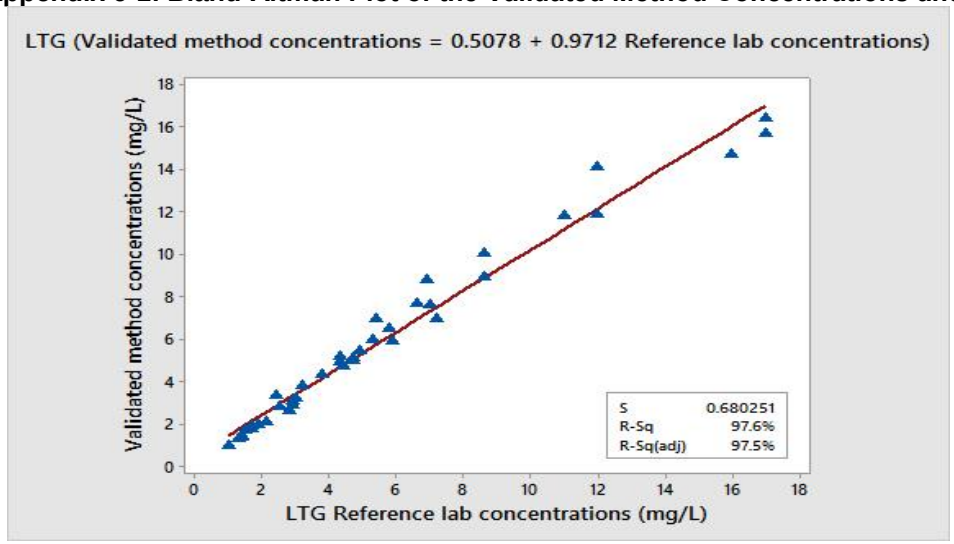


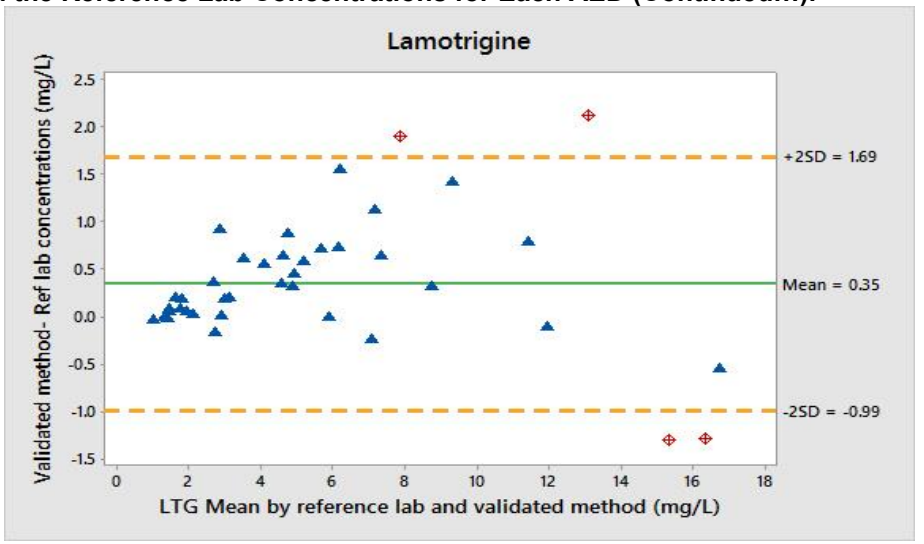


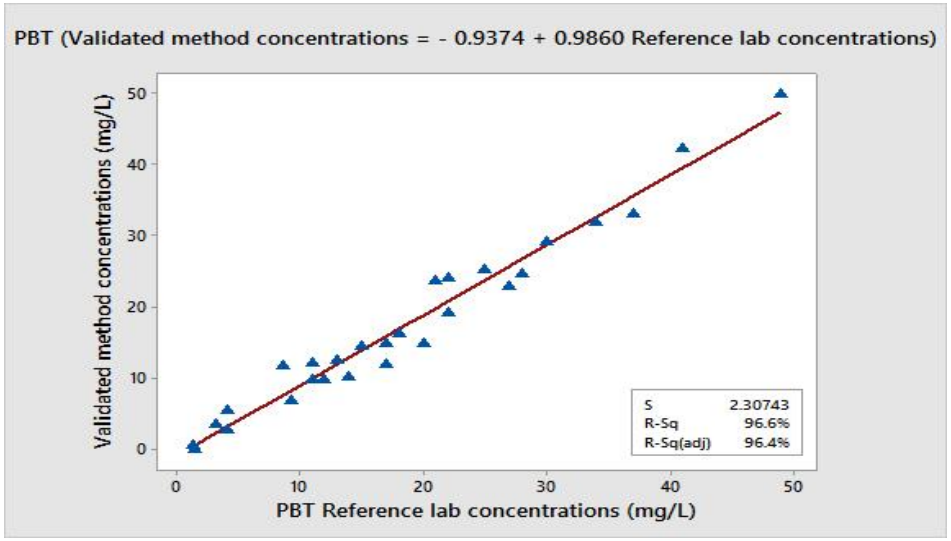


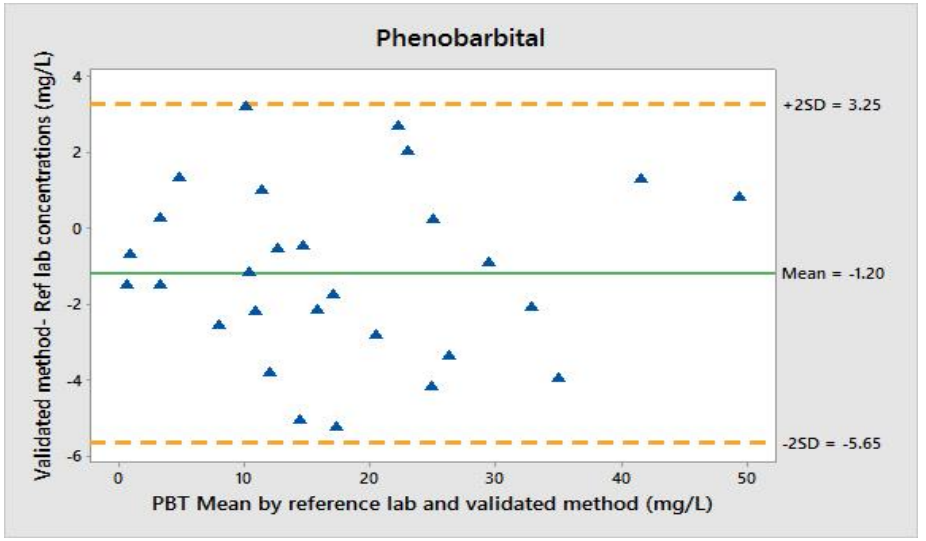


Appendix 6-1: Bland-Altman Plot of the Validated Method Concentrations and the Reference Lab Concentrations for Each AED (Continued…).

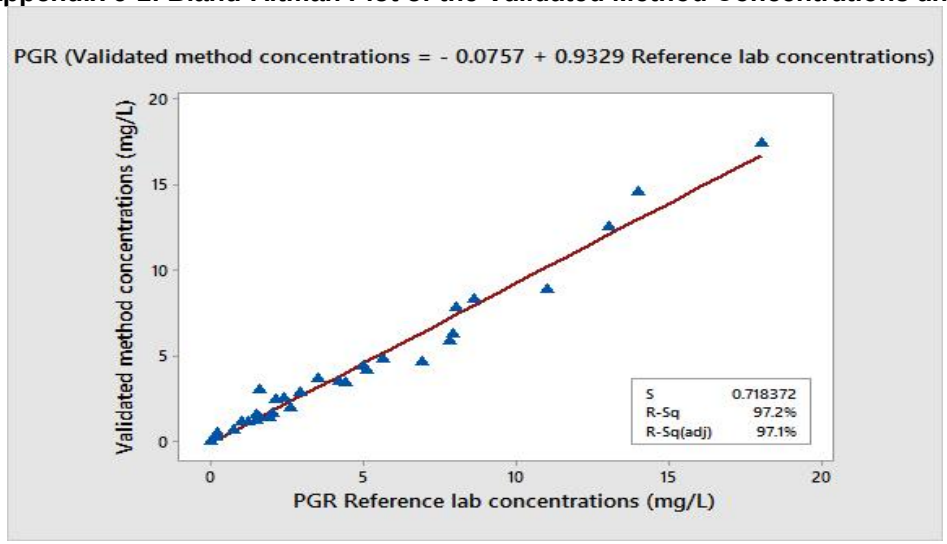


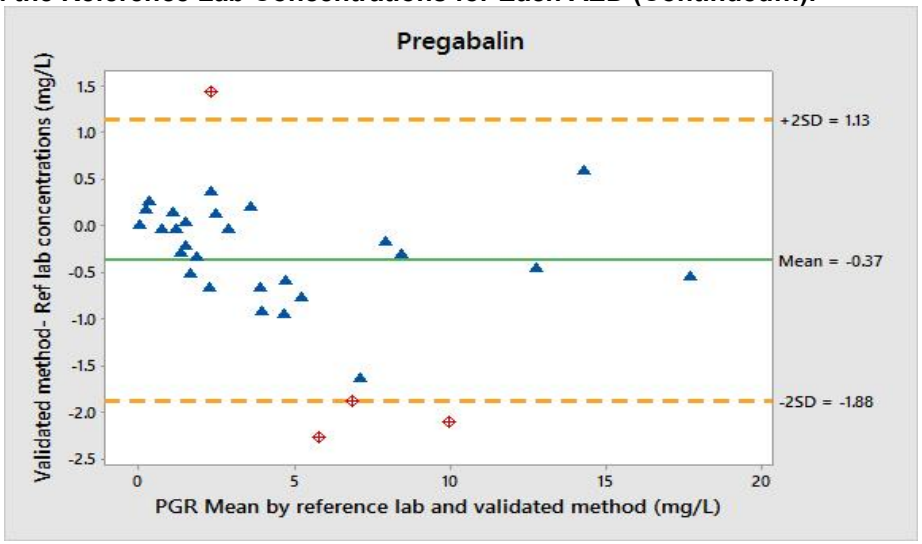


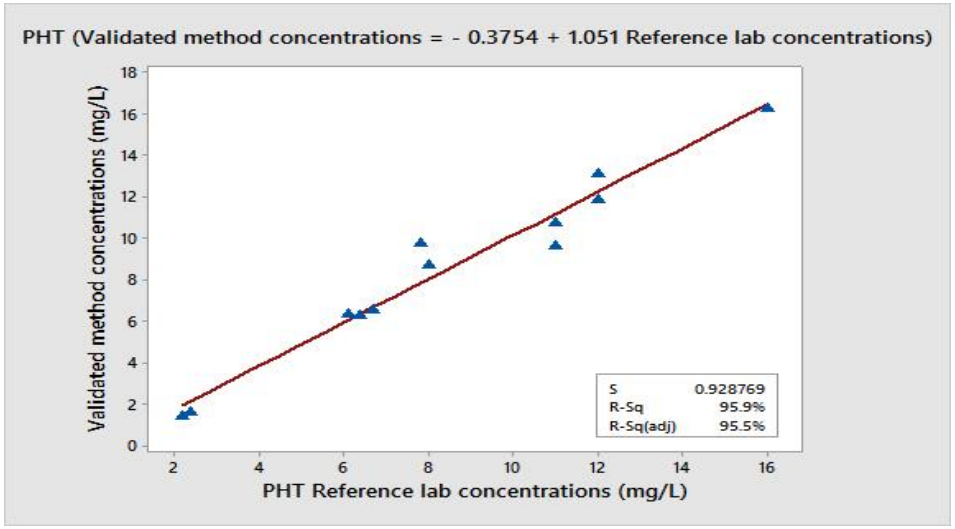


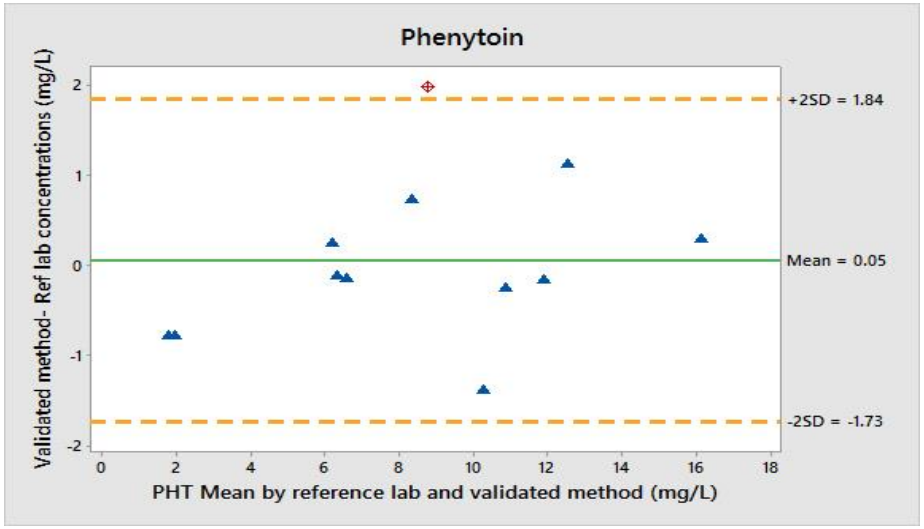


Appendix 6-1: Bland-Altman Plot of the Validated Method Concentrations and the Reference Lab Concentrations for Each AED (Continued…).

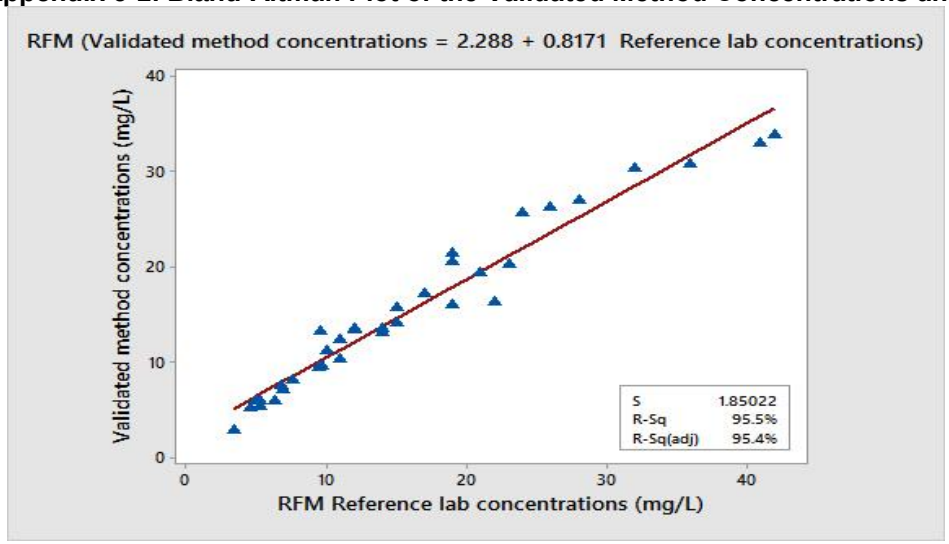


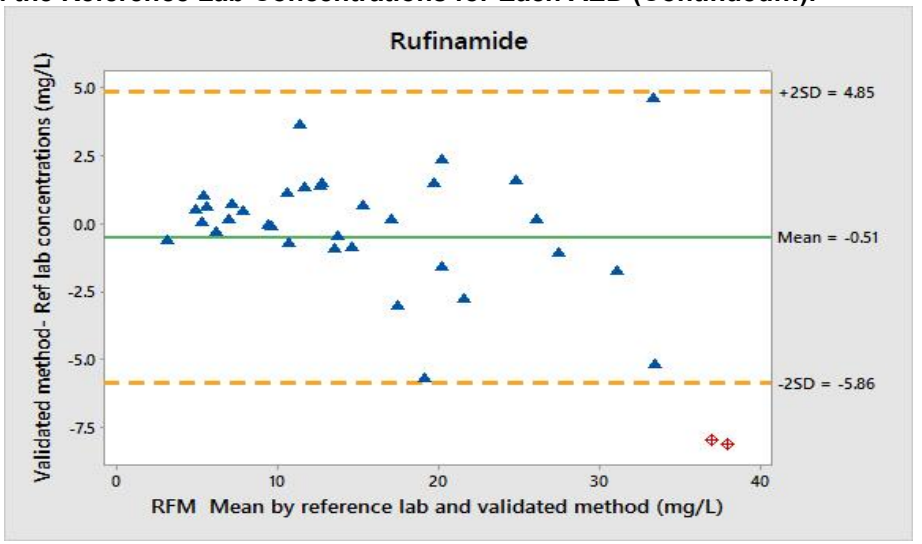


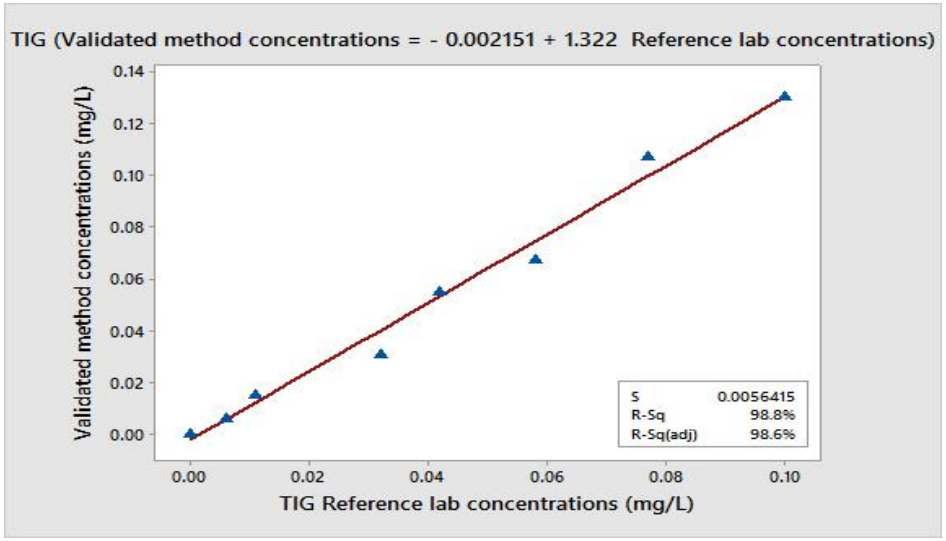


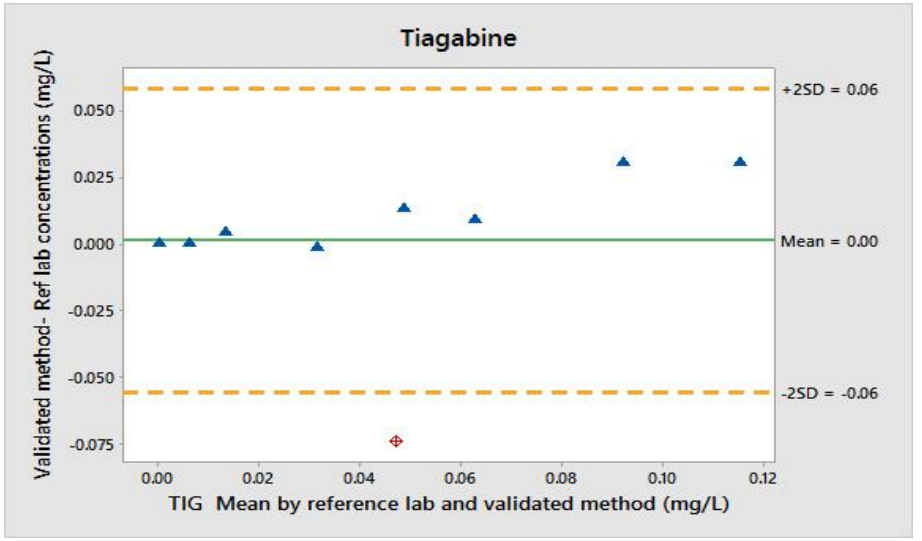


Appendix 6-1: Bland-Altman Plot of the Validated Method Concentrations and the Reference Lab Concentrations for Each AED (Continued…).

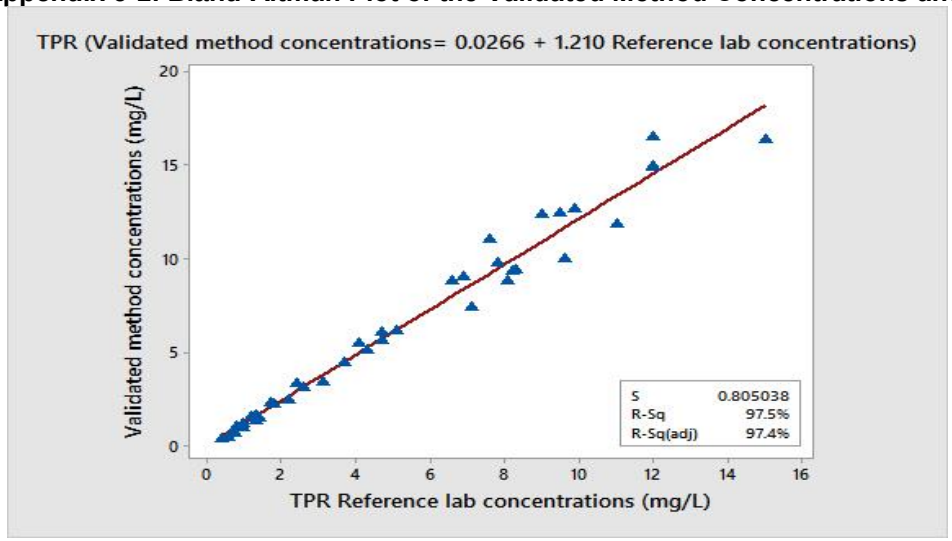


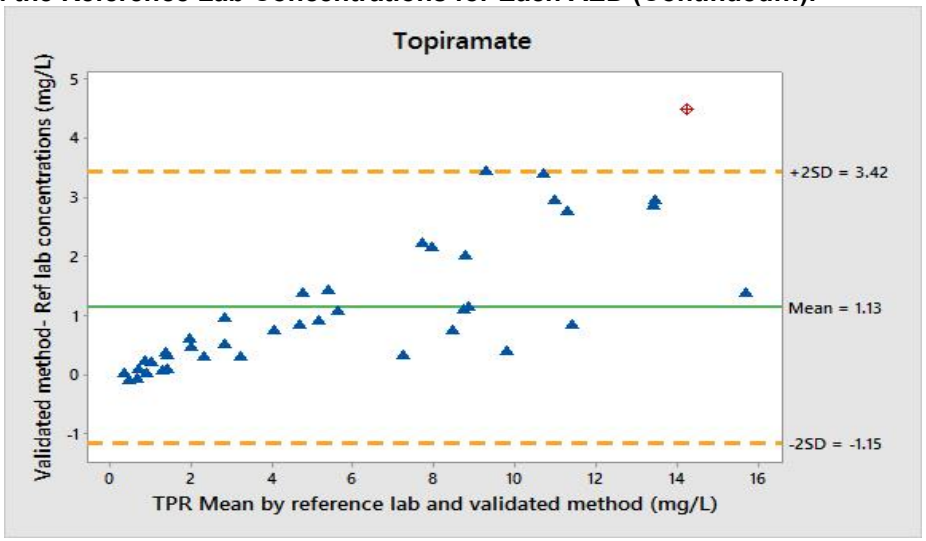


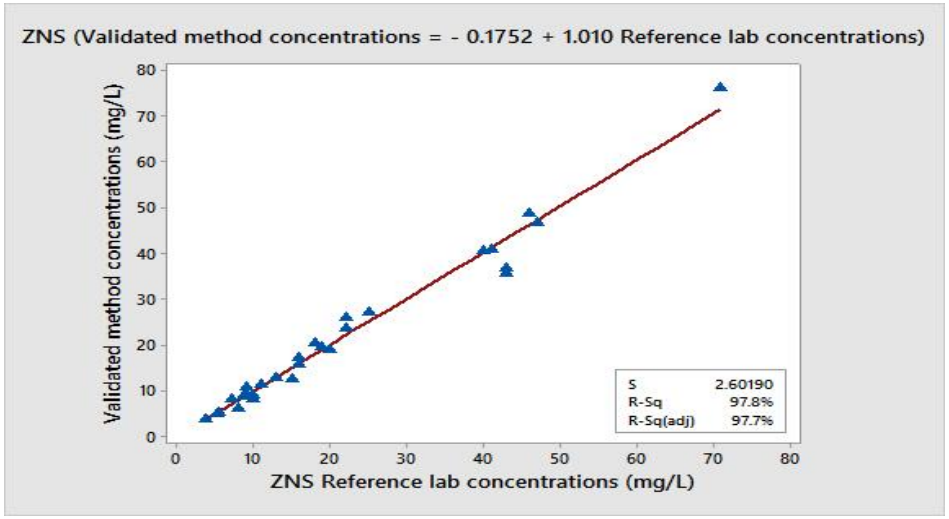


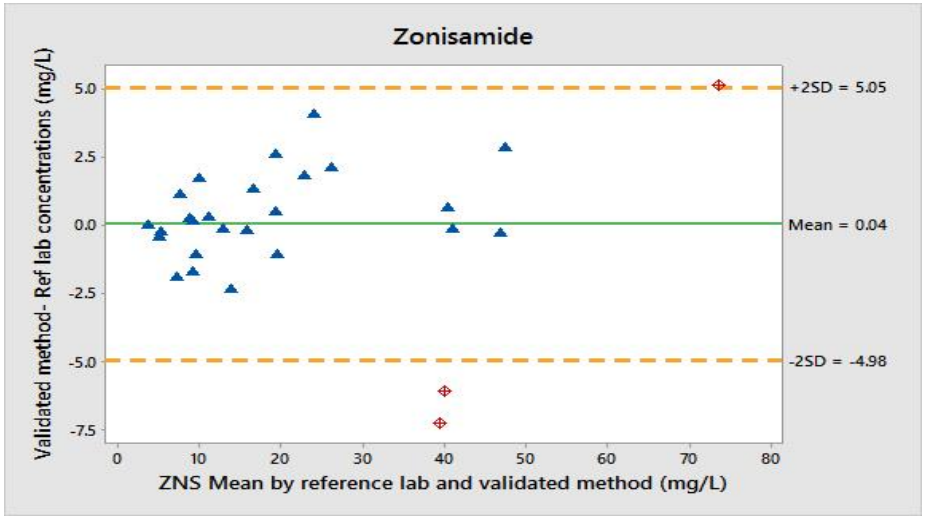


Appendix 6-1: Bland-Altman Plot of the Validated Method Concentrations and the Reference Lab Concentrations for Each AED (Continued…).









Appendix 7-1: Ethical Approval and Amendment Letters from the West of Scotland Research Ethics Service (WoSRES) to Collect Urine Samples from Prisoners.

WoSRES
West of Scotland Research Ethics Service

NHS Greater Glasgow and Clyde

West of Scotland REC 4
Ground Floor, Tennent Building
Western Infirmary
38 Church Street
Glasgow
G11 6NT
www.nhsggc.org.uk

Mrs Shaza Deeb
Forensic Medicine and Science
University of Glasgow
University Place
Glasgow
G12 8QQ

Date 14 December 2012
Direct line 0141-211-1722
Fax 0141-211-1847
e-mail evelyn.jackson@ggc.scot.nhs.uk

Dear Mrs Deeb

Study title:	**Determination of New Antiepileptic Drugs in Human Samples (Urine and Head Hair) Using Chromatographic Techniques Coupled with Mass Spectrometry As Prospective Drugs of Misuse**
REC reference:	**12/WS/0312**
Protocol number:	**1.0**
IRAS project ID:	**104688**

The Research Ethics Committee reviewed the above application at the meeting held on 7 December 2012. Thank you for attending to discuss the application.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Ms Evelyn Jackson, evelyn.jackson@ggc.scot.nhs.uk.

Ethical opinion

1. The CI explained that the anti-epilepsy drugs mentioned in the application are easily available and are being sold as painkillers or for anti-depressants, from many sources. Information from the Scottish Crime and Drug Enforcement Agency suggests that these drugs are beginning to be abused in Scotland in much the same way as diazapam and there is evidence that prisoners are abusing this drug.

2. The Committee were unclear if the prisoners participating in the study would already have provided a urine sample to the Addiction Team in the prison.

   The CI explained that new prisoners routinely had to provide a urine sample when they were admitted into Barlinnie Prison and that the CI hoped to take a residual sample from this.

3. The Committee noted that hair samples would be taken from 200 prisoners in Barlinnie Prison and urine samples would be taken from 100 people detained in the Police office and wondered why this had been decided and urine and hair samples would deliver equally good results.

   The CI explained that she had been advised by prison officers that it would be difficult to obtain hair samples from the prison population, but felt confident that hair and urine would provide the equal results.

4. The Committee asked if a patient was taking other medication would this affect the result of the AED.

   The CI explained that a screen for the samples had been developed which would detect if any anti-epileptic had been taken, therefore it would matter to the result if any other drug had been taken.

Decison: Favourable Opinion with additional conditions

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

The Committee gave a favourable opinion of the study with the following conditions:

1. In the Information Sheets:
(a) Make it explicitly clear at the outset that any samples taken for use in the study would be completely anonymous.
(b) In the section headed "What is the purpose of the study?", give a clearer explanation as to the purpose of the study.
(c) In the section headed "Who has reviewed the study?", give the name of the REC - West of Scotland Research Ethics Committee 4".
(d) Part I and Part II should be combined to make a one-part document.

2. In the Donor Information - Police Station, add a question asking if the hair has been dyed.

The Committee nominated the Co-ordinator to be the point of contact should further clarification be sought by the researcher upon receipt of the decision letter.

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
REC application	-	23 November 2012

Protocol	1.0	20 November 2012
Investigator CV	-	08 August 2012
Participant Information Sheet: Police Station	1.0	20 November 2012
Participant Information Sheet: Prison	1.0	20 November 2012
Participant Consent Form: Police Station	1.0	20 November 2012
Participant Consent Form: Prison	1.0	20 November 2012
Evidence of insurance or indemnity	Police	18 July 2012
Evidence of insurance or indemnity	Prison	18 July 2012

Other: Karen Scott's CV	-	06 August 2012
Other: Poster - Police Station	1.0	20 November 2012
Other: GP Surgeries and Participants Instructions - Police Station	1.0	20 November 2012
Other: GP Surgery and Participants Instructions - Prison	1.0	20 October 2012
Other: Donor Information - Prison	1.0	20 November 2012
Other: Donor Information - Police Station	1.0	20 November 2012

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/WS/0312	Please quote this number on all correspondence

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

Evelyn Jackson

***For* Dr Brian Neilly Chair**

Enclosures: *List of names and professions of members who were present at the meeting*
"After ethical review – guidance for researchers"

Copy to: *Dr Karen S Scott*
Dr Oliver Frenschock

West of Scotland REC 4

Attendance at Committee meeting on 07 December 2012

Committee Members:

Name	*Profession*	*Present*	*Notes*
Ms Lynda Brown	Public Health Adviser	No	
Dr Andrew Clark	Consultant Haematologist	No	
Ms Cristina Coelho	Pharmacist	No	
Dr Clair Evans	Consultant Paediatric and Perinatal Pathologist	No	
Dr Kenneth James	Consultant Anaesthetist	No	
Dr Grace Lindsay	Reader	Yes	
Miss Fiona Mackelvie	(Retired) Lay member	Yes	
Ms Margaret McDonald	Retired (Lay Member)	No	
Mrs Cynthia Mendelsohn	Retired (Lay member)	Yes	
Dr Brian Neilly	Consultant Physician	Yes	
Dr Jackie Riley	Statistician	Yes	
Mrs Kathleen Tuck	Retired Teacher	No	
Mr Iain Wright	Consultant Engineer (Lay member)	Yes	
Dr Ihab Shaheen	Consultant Paediatrician	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Dr Judith Godden	Scientific Adviser
Ms Evelyn Jackson	Committee Co-ordinator

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West of Scotland Research Ethics Service

NHS
Greater Glasgow and Clyde

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Mrs Shaza Deeb
PhD Student
University of Glasgow
Forensic Medicine and Science
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University Place
Glasgow
G12 8QQ

Date 21 December 2012
Direct line 0141-211-1722
Fax 0141-211-1847
e-mail evelyn.jackson@ggc.scot.nhs.uk

Dear Mrs Deeb

Study title:	**Determination of New Antiepileptic Drugs in Human Samples (Urine and Head Hair) Using Chromatographic Techniques Coupled with Mass Spectrometry As Prospective Drugs of Misuse.**
REC reference:	**12/WS/0312**
Protocol number:	**1.0**
IRAS project ID:	**104688**

Thank you for your e-mail of 19 December 2012. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 14 December 2012

Documents received

The documents received were as follows:

Document	Version	Date
Covering Letter	e-mail	19 December 2012
Other: Donor Information - Police Station	1.1	19 December 2012
Participant Information Sheet: Prison	1.1	19 December 2012
Participant Information Sheet: Police Station	1.1	19 December 2012

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	Version	Date
Covering Letter	e-mail	19 December 2012
REC application	-	23 November 2012
Protocol	1.0	20 November 2012
Participant Information Sheet: Prison	1.1	19 December 2012
Participant Information Sheet: Police Station	1.1	19 December 2012
Participant Consent Form: Police Station	1.0	20 November 2012
Participant Consent Form: Prison	1.0	20 November 2012
Evidence of insurance or indemnity	Police	18 July 2012
Evidence of insurance or indemnity	Prison	18 July 2012
Investigator CV	-	08 August 2012
Other: Karen Scott's CV	-	06 August 2012
Other: Poster - Police Station	1.0	20 November 2012
Other: GP Surgeries and Participants Instructions - Police Station	1.0	20 November 2012
Other: GP Surgery and Participants Instructions - Prison	1.0	20 October 2012
Other: Donor Information - Prison	1.0	20 November 2012
Other: Donor Information - Police Station	1.1	19 December 2012

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

12/WS/0312 **Please quote this number on all correspondence**

Yours sincerely

Evelyn Jackson

Ms Evelyn Jackson
Committee Co-ordinator

Copy to: *Dr Karen S Scott,*
Dr Oliver Frenschock

WoSRES
West of Scotland Research Ethics Service

NHS
Greater Glasgow and Clyde

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Mrs Shaza Deeb
PhD Student
University of Glasgow
Forensic Medicine and Science
University Place
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G12 8QQ

Date 11 February 2013
Direct line 0141-211-1722
Fax 0141-211-1847
e-mail evelyn.jackson@ggc.scot.nhs.uk

Dear Mrs Deeb

Study title:	**Determination of New Antiepileptic Drugs in Human Samples (Urine and Head Hair) Using Chromatographic Techniques Coupled with Mass Spectrometry As Prospective Drugs of Misuse**
REC reference:	**12/WS/0312**
Amendment number:	**AM01**
Amendment date:	**08 February 2013**
IRAS project ID:	**104688**

Thank you for your letter of 8 February 2013, notifying the Committee of the following amendments:

- Falkirk Police Office added as an additional non-NHS site.
- Sponsor's contact changed to Dr Debra Stuart.

The Committee does not consider this to be a "substantial amendment", as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received

The documents received were as follows:

Document	Version	Date
Notification of a Minor Amendment	AM01	08 February 2013

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

12/WS/0312:	Please quote this number on all correspondence

Yours sincerely

Ms Evelyn Jackson
Committee Co-ordinator

Copy to: *Dr Oliver Frenschock*
Dr D Stuart, University of Glasgow, Tennent Building

WoSRES
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Mrs Shaza Deeb
PhD Student
University of Glasgow
Forensic medicine and Science
University of Glasgow
University Place
Glasgow
G12 8QQ

Dear Mrs Deeb

Study title:	**Determination of New Antiepileptic Drugs in Human Samples (Urine and Head Hair) Using Chromatographic Techniques Coupled with Mass Spectrometry As Prospective Drugs of Misuse.**
REC reference:	**12/WS/0312**
Amendment number:	**AM02**
Amendment date:	**23 May 2013**
IRAS project ID:	**104688**

Thank you for your letter of 23 May 2013, notifying the Committee of the following amendment:

- Additional non-NHS site – HMP Cornton Vale, Stirling.

The Committee does not consider this to be a "substantial amendment", as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received

The documents received were as follows:

Document	Version	Date
Notification of a Minor Amendment	AM02	23 May 2013

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

Appendix 7-2: Details of Illicit Drugs Detected in Positive AEDs Urine Sample.

Sample No.	Prison	Sample[a] Type	GBP	PGR	Other AEDs	AMP[b]	OPI[c]	BNZ[d]	CAN[e]	COC[f]	MAMP[g]	BARB[h]	BUP[i]	MTD[j]	Prescribed Medication
1	Low Moss	A	+												
2	Low Moss	A	+	+			+	+	+		+			+	
3	Low Moss	A	+						+						
4	Low Moss	A	+					+	+	+					
5	Low Moss	A			CBZ			+	+						
6	Low Moss	A			VPA LEV			+	+						
7	Low Moss	A	+					+	+					+	
8	Low Moss	A		+		+	+	+	+	+	+	+	+	+	
9	Low Moss	A	+				+	+						+	
10	Low Moss	A	+				+	+							
11	Low Moss	A	+				+	+	+						
12	Low Moss	A			CBZ		+	+	+	+				+	
13	Low Moss	A	+	+			+	+	+			+			
14	Low Moss	A	+					+	+		+				
15	Low Moss	A	+					+							
16	Low Moss	A	+				+								
17	Low Moss	A	+				+	+							
18	Low Moss	A	+				+	+		+				+	
19	Low Moss	A	+	+			+	+		+				+	
20	Low Moss	L	+		TPR										
21	Addiwell	A	+				+	+							
22	Addiwell	A	+					+							

[a]A: Admission and L: Liberation, [b]AMP: Amphetamines, [c]OPI: Opiates, [d]BNZ: Benzodiazepines, [e]CAN: Cannabis, [f]COC: Cocaine, [g]MAMP: Methamphetamine, [h]BARB: Barbiturates, [I]BUP: Buprenorphine, [j]MTD: Methadone.

Appendix 7-2: Details of Illicit Drugs Detected in Positive AEDs Urine Sample (Continued…).

Sample No.	Prison	Sample Type	GBP	PGR	Other AEDs	AMP	OPI	BNZ	CAN	COC	MAMP	BARB	BUP	MTD	Prescribed Medication
23	Addiwell	A	+												
24	Addiwell	A	+					+	+				+		Suboxone
25	Addiwell	A	+					+	+	+			+		
26	Addiwell	A	+				+		+				+		
27	Addiwell	A	+				+	+					+		Suboxone
28	Addiwell	A	+				+	+	+				+	+	
29	Addiwell	A	+				+	+	+				+	+	
30	Addiwell	A	+					+	+						
31	Addiwell	A	+	+					+						
32	Addiwell	L	+											+	Methadone
33	Addiwell	L	+					+							
34	Addiwell	L		+											
35	Addiwell	L	+										+		
36	Corton Vale	L		+										+	Methadone
37	Corton Vale	L		+										+	Methadone
38	Corton Vale	L	+	+			+							+	Methadone
39	Corton Vale	A	+					+							
40	Corton Vale	A	+				+	+	+						
41	Corton Vale	A	+				+	+		+				+	
42	Corton Vale	A	+			+	+	+	+					+	Methadone
43	Corton Vale	A	+				+	+					+	+	Dihydrocodeine, Diazapam, Methadone

[a]A: Admission and L: Liberation, [b]AMP: Amphetamines, [c]OPI: Opiates, [d]BNZ: Benzodiazepines, [e]CAN: Cannabis, [f]COC: Cocaine, [g]MAMP: Methamphetamine, [h]BARB: Barbiturates, [I]BUP: Buprenorphine, [j]MTD: Methadone.

Appendix 7-2: Details of Illicit Drugs Detected in Positive AEDs Urine Sample (Continued…).

Sample No.	Prison	Sample Type	GBP	PGR	Other AEDs	AMP	OPI	BNZ	CAN	COC	MAMP	BARB	BUP	MTD	Prescribed Medication
44	Corton Vale	A	+					+	+					+	Methadone
45	Corton Vale	A			VPA		+	+	+				+	+	
46	Corton Vale	A		+			+	+	+					+	Methadone
47	Corton Vale	A	+				+	+	+					+	Methadone
48	Corton Vale	A		+			+	+						+	Methadone
49	Corton Vale	A			LTG			+					+		Diazapam
50	Corton Vale	A	+					+							
51	Corton Vale	A	+				+		+			+		+	Zoplicone, Methadone
52	Corton Vale	A	+				+	+	+						
53	Corton Vale	A	+												
54	Corton Vale	A	+				+	+	+	+					
55	Corton Vale	A	+				+	+	+	+			+		Diazapam
56	Corton Vale	A	+				+	+	+				+		
57	Corton Vale	A	+	+				+						+	Methadone, Diazepam
58	Corton Vale	A	+				+	+	+	+				+	Methadone
59	Corton Vale	A	+				+	+					+		Diazepam
60	Corton Vale	A	+				+	+		+				+	
61	Corton Vale	A	+				+								Dihydrocodeine
62	Corton Vale	A	+				+	+	+					+	
63	Barlinnie	L	+				+								Co-codamol
64	Barlinnie	L	+											+	Methadone
65	Barlinnie	L	+											+	Methadone

[a]A: Admission and L: Liberation, [b]AMP: Amphetamines, [c]OPI: Opiates, [d]BNZ: Benzodiazepines, [e]CAN: Cannabis, [f]COC: Cocaine, [g]MAMP: Methamphetamine, [h]BARB: Barbiturates, [I]BUP: Buprenorphine, [j]MTD: Methadone.

Appendix 7-2: Details of Illicit Drugs Detected in Positive AEDs Urine Sample (Continued…).

Sample No.	Prison	Sample Type	GBP	PGR	Other AEDs	AMP	OPI	BNZ	CAN	COC	MAMP	BARB	BUP	MTD	Prescribed Medication
66	Barlinnie	L	+											+	Methadone
67	Barlinnie	L		+											
68	Barlinnie	L	+					+						+	Methadone, Diazapam
69	Barlinnie	A	+				+	+	+	+				+	Co-codamol, Methadone
70	Barlinnie	A	+										+		
71	Barlinnie	A	+				+	+		+			+		
72	Barlinnie	A	+				+	+	+	+				+	Methadone, Diazepam
73	Barlinnie	A	+				+	+						+	Methadone
74	Barlinnie	A	+				+	+	+	+				+	Methadone
75	Barlinnie	A	+					+							
76	Polmont	L		+			+	+	+				+		Suboxone
77	Polmont	L		+											
78	Polmont	L		+					+						
79	Polmont	A	+						+						
80	Polmont	A	+					+	+						
81	Polmont	L			LEV										
82	Polmont	A	+					+	+						
83	Polmont	A			LEV										

[a]A: Admission and L: Liberation, [b]AMP: Amphetamines, [c]OPI: Opiates, [d]BNZ: Benzodiazepines, [e]CAN: Cannabis, [f]COC: Cocaine, [g]MAMP: Methamphetamine, [h]BARB: Barbiturates, [I]BUP: Buprenorphine, [j]MTD: Methadone.

Appendix 7-2: Details of Illicit Drugs Detected in Positive AEDs Urine Sample (Continued…).

Sample No.	Prison	Sample Type	GBP	PGR	Other AEDs	AMP	OPI	BNZ	CAN	COC	MAMP	BARB	BUP	MTD	Prescribed Medication
84	Perth	A	+			+	+	+	+	+		+	+		
85	Perth	A			VIG			+	+	+					
86	Perth	A	+												
87	Perth	A		+											
88	Perth	A	+					+	+					+	
89	Perth	A			LTG		+		+				+	+	
90	Perth	A	+	+			+	+						+	
91	Perth	A	+	+			+	+							
92	Perth	A	+				+	+	+					+	
93	Perth	A	+				+		+						
94	Perth	A	+				+	+	+				+		
95	Perth	A	+				+	+	+						
96	Perth	A	+				+	+						+	
97	Perth	A	+				+	+							
98	Perth	A	+				+							+	
99	Perth	A	+				+	+	+						
100	Perth	A	+				+	+						+	
101	Perth	A	+				+	+		+			+	+	
102	Perth	A	+				+	+	+					+	
103	Perth	A			VIG										
104	Perth	A	+				+	+	+					+	

[a]A: Admission and L: Liberation, [b]AMP: Amphetamines, [c]OPI: Opiates, [d]BNZ: Benzodiazepines, [e]CAN: Cannabis, [f]COC: Cocaine, [g]MAMP: Methamphetamine, [h]BARB: Barbiturates, [I]BUP: Buprenorphine, [j]MTD: Methadone.

Appendix 7-2: Details of Illicit Drugs Detected in Positive AEDs Urine Sample (Continued…).

Sample No.	Prison	Sample Type	GBP	PGR	Other AEDs	AMP	OPI	BNZ	CAN	COC	MAMP	BARB	BUP	MTD	Prescribed Medication
105	Perth	A	+				+	+						+	
106	Perth	A	+		LTG		+	+						+	
107	Perth	A	+				+	+	+			+		+	
108	Perth	A	+				+	+	+	+	+			+	
109	Perth	A	+				+	+	+						
110	Perth	A	+				+	+	+						
111	Perth	A			VPA	+	+	+			+		+		
112	Perth	L		+			+							+	
113	Perth	L	+												
114	Perth	L	+											+	
115	Perth	L	+											+	
116	Perth	L		+										+	
117	Perth	L	+			+	+		+	+				+	

[a]A: Admission and L: Liberation, [b]AMP: Amphetamines, [c]OPI: Opiates, [d]BNZ: Benzodiazepines, [e]CAN: Cannabis, [f]COC: Cocaine, [g]MAMP: Methamphetamine, [h]BARB: Barbiturates, [I]BUP: Buprenorphine, [j]MTD: Methadone.

Appendix 9: Papers and Awards in Support of This Thesis.

Publications

- Shaza Deeb, Denise A. McKeown, Hazel J. Torrance, Fiona M. Wylie, Barry K. Logan, Karen S. Scott. "Simultaneous Analysis of 22 Antiepileptic Drugs in Postmortem Blood, Serum and Plasma Using LC-MS-MS with a Focus on Their Role in Forensic Cases". ***Journal of Analytical Toxicology-Special edition***, 2014, 38 (8): 485-494.

Oral presentation

- Shaza Deeb, Fiona M. Wylie, Karen S. Scott. "Gabapentin and Pregabalin prevalence among prisoners in Scotland - an insight into their abuse potential".

  - Presented at The United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT) meeting, ***Aberdeen, UK (August 2014).***

  - Presented at the Scottish Prison Service, ***Edinburgh, UK (April 2015)***

- Karen S. Scott, Shaza Deeb. "Determination of 22 antiepileptic drugs in postmortem blood, serum and plasma using LC/MS/MS with a focus on their role in forensic cases". Presented at the Eastern Analytical Symposium & Exposition, ***Somerset, United States (November 2014).***

- Shaza Deeb, Fiona M. Wylie, Barry K. Logan, Karen S. Scott. "Determination of 22 antiepileptic drugs in postmortem blood, serum and plasma using LC/MS/MS with a focus on their role in forensic cases". Presented at NMS Lab as part of their continuing education course, ***Philadelphia, United States (April 2014).***

Posters

- Shaza Deeb, Denise A. McKeown, Hazel J. Torrance, Fiona M. Wylie, Karen S. Scott. "Extraction optimization of 15 antiepileptic drugs and two selected metabolites in postmortem whole blood using LC/MS/MS". Presented at and in Proceedings of Novel Psychoactive Substance (NPS) Conference, ***Swansea, UK (September 2013).***

- Shaza Deeb, Denise A. McKeown, Hazel J. Torrance, Fiona M. Wyli,; Barry K. Logan,; Karen S. Scott. "Simultaneous analysis of 17 antiepileptic drugs in whole blood using LC/MS/MS with a focus on their role in forensic cases".

  - Presented at and in proceedings of SOFT conference, ***Orlando, FL, USA, (October 2013).***

  - Presented at AAFS conference, ***Seattle, USA (February 2014).***

- Eleanor M. Berry, Denise A. McKeown, Hazel J. Torrence, Shaza Deeb. "Significance of pregabalin and gabapentin concentrations in postmortem blood".

  - Presented at The United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT) meeting, ***Leicester, UK (August 2014).***

Award:

- **Experimental Design and Impact on Toxicology (EDIT) Award** - Society of Forensic Toxicologists (SOFT), Annual Business Meeting, Michigan, USA (October 2014).

  Shaza Deeb, Denise A. McKeown, Hazel J. Torrance, Fiona M. Wylie, Barry K. Logan, Karen S. Scott. "Simultaneous Analysis of 22 Antiepileptic Drugs in Postmortem Blood, Serum and Plasma Using LC-MS-MS with a Focus on Their Role in Forensic Cases". ***Journal of Analytical Toxicology-Special edition***, 2014, 38 (8): 485-494.

Journal of Analytical Toxicology 2014;38:485–494
doi:10.1093/jat/bku070

Special Issue

Simultaneous Analysis of 22 Antiepileptic Drugs in Postmortem Blood, Serum and Plasma Using LC–MS-MS with a Focus on Their Role in Forensic Cases

Shaza Deeb[1]*, Denise A. McKeown[1], Hazel J. Torrance[1], Fiona M. Wylie[1], Barry K. Logan[2] and Karen S. Scott[3]

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In recent years, there has been a growth in reports of antiepileptic drugs (AEDs) being misused on their own or in combination with other drugs of abuse in a variety of toxicological case types such as drug abuse, suicide, overdose and drug facilitated crime. To our knowledge, there are no simultaneous quantification methods for the analysis of the most commonly encountered AEDs in postmortem whole blood and clinical plasma/serum samples at the same time. A simple, accurate and cost-effective liquid chromatography–tandem mass spectrometric (LC–MS-MS) method has been developed and validated for the simultaneous quantification of carbamazepine (CBZ) and its metabolite CBZ-10,11-epoxide, eslicarbazepine acetate, oxcarbazepine and *S*-licarbazepine as a metabolite, gabapentin, lacosamide, lamotrigine, levetiracetam, pregabalin, phenobarbital, phenytoin and its metabolite 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, retigabine (ezogabine) and its metabolite *N*-acetyl retigabine, rufinamide, stiripentol, topiramate, tiagabine, valproic acid, vigabatrin and zonisamide in postmortem whole blood, serum and plasma which would be suitable for routine forensic toxicological analysis and therapeutic drug monitoring. All AEDs were detected and quantified within 17 min without endogenous interferences. The correlation coefficient (R^2) was >0.995 for all AEDs with accuracy ranging from 90 to 113% and precision <13% for all analytes. The recovery ranged from 70 to 98%. No carryover was observed in a blank control injected after the highest standard and the matrix effect was acceptable and ranged from 90 to 120%. The method has been successfully verified using authentic case samples that had previously been quantified using different methods.

Introduction

In recent years, antiepileptic drugs (AEDs) have been associated with an increased likelihood of off-label prescription in non-epilepsy disorders even though most of these indications are still under investigation (1). Between 2004 and 2007, the use of newer AEDs increased from 40 to 49% in epilepsy, whereas it rose from 5 to 64% in psychiatry mainly due to an increase in the number of lamotrigine (LTG) prescriptions; from 79 to 95% in neuropathic pain as a result of the large increase in pregabalin (PGR) prescriptions; and from 3 to 16% in trigeminal neuralgia where PGR and gabapentin (GBP) dominated (2). The widespread use of the new generation of AEDs, particularly in patients with psychiatric disorders, often for unlicensed indications, increases the risk of self-poisoning (3), suicide (4) and drug abuse (5–7). AEDs are a group of legal prescription drugs, which are found in abuse cases either alone or with alcohol or other common drugs of abuse in order to enhance their effects (8, 9). Drugs fitting this description include GBP (10) and PGR (11).

Sudden unexplained death in epilepsy (SUDEP) is a major cause of death among epileptic patients. It is responsible for 18% of epileptic-related deaths (12). The second most important factor after the frequency of seizures is the number of AEDs taken concomitantly. The risk is almost 10 times higher in patients taking more than two drugs compared with those who are on monotherapy (12, 13). Furthermore, many of the AEDs might impair driving if their concentrations are not maintained properly; therefore, this group of drugs should be tested in cases of suspected impaired driving (14, 15).

There are many reasons for applying therapeutic drug monitoring (TDM) to AEDs. A common reason is that the pharmacokinetics of the drug shows significant inter-individual variability. It is therefore important to ensure that an individual's plasma concentration is within the therapeutic range. A number of factors may alter serum protein concentrations of AEDs including liver disease, old age and pregnancy. Concomitant medications or endogenous substances may displace drugs from serum protein-binding sites, potentially leading to higher free drug concentrations, higher side effects (drowsiness, sedation) and less effectiveness (16). Various analytical tools have been developed for TDM of AEDs (17, 18). Automated immunoassay methods have been the most widely used methods for the determination of anticonvulsants for TDM since the 1980s (19).

A number of simultaneous chromatographic assays for AEDs have been developed using GC (20) and HPLC coupled to ultraviolet detection (21) or an evaporative light-scattering detector (22). The initial simultaneous assays, from the 1980s, concentrated on separating the older AEDs such as ethosuximide, primidone, carbamazepine (CBZ), CBZ-epoxide, phenytoin (PHT) and phenobarbital (PBT) with the inclusion or removal of one or more additional drugs or metabolites such as ethyl-phenacemide, 5-*para*-hydroxyphenyl-5-phenylhydantoin, *N*-des-methyl-methsuximide, phenyl-2-ethyl-malonamide and LTG (23–25). However, these methods required time-consuming and difficult extraction procedures or relatively large sample volumes (~1 mL) as well as lengthy chromatographic run times, which limited their throughput capacity and sensitivity. In addition, all methods which employ ultraviolet detection have the risk of interferences due to matrix, metabolites or other co-medications (26) and they are not applicable to some AEDs due to a lack of a chromophore such as PGR, GBP, vigabatrin (VIG), topiramate (TPR) and valproic acid (VPA) (27, 28).

Several methods have been described for the simultaneous LC–MS-MS analysis of a selection of AEDs in one analytical step (29–31). All of these methods were designed for TDM in plasma/serum over the therapeutic ranges. None of these methods were validated for postmortem blood, plasma and serum over a wide concentration range including those associated with toxicity levels.

The aim of this project was to develop and validate a quantification method for the analysis of 18 AEDs and four metabolites in postmortem whole blood, serum and plasma, which would be

suitable for routine forensic toxicological analysis and TDM at the same time.

Materials and methods

Chemicals and reagents

Lacosamide (LAC), PGR and tiagabine (TIG) were obtained from LGC standards. Eslicarbazepine acetate (ESL) was purchased from Santa Cruze Biotechnology. *S*-Licarbazepine (*S*-LC), ezogabine (RTG), *N*-acetyl ezogabine (NA-RTG), PBT, rufinamide (RFM) and gabapentin -D_{10} (GBP-D_{10}) were purchased from Cerilliant. GBP, VIG, VPA, levetiracetam (LEV), CBZ, CBZ 10,11-epoxide (CBZO), oxcarbazepine (OXC), zonisamide (ZNS), TPR, PHT, 5-(3-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), stiripentol (STP), LTG, tolbutamide (TUB), 10,11 dihydrocarbamazepine (CBZ-DiOH) and ammonium acetate (HPLC grade) were purchased from Sigma–Aldrich. Methanol (HPLC grade) was supplied by VWR International Ltd. Double-distilled water was obtained from the in-house Millipore® system.

Calibrators, quality control and internal standards preparation

Stock solutions for AEDs were prepared in methanol for LEV, VPA and VIG at 10 and 1 mg/mL for all other drugs. Three working solutions were prepared by further diluting the stock solutions in methanol to obtain 1 mg/mL for LEV, VPA and VIG; 20 μg/mL for RTG, NA-RTG, TIG and OXC and 100 μg/mL for the rest of the drugs. Eight calibration standard solutions were prepared in methanol from the working solutions to achieve the target concentrations.

Three internal standard stock solutions of GBP-D_{10}, TUB and CBZ-DiOH were prepared in methanol to give a concentration of 10 μg/mL. A combined working internal standard solution was prepared in methanol at 2.5 μg/mL for GBP-D_{10} and 5 μg/mL for TUB and CBZ-DiOH. Three quality control (QC) samples (low, medium and high) were directly made in whole blood, plasma and serum. QC levels were 40, 120 and 200 mg/L for LEV, VIG and VPA; 2, 4 and 9 mg/L for OXC, TIG, NA-RTG and RTG and 3, 20 and 40 mg/L for all the other AEDs. All QCs and stock solutions were stored at −20°C, and working solutions were stored at 4°C.

Calibration curves were extracted in duplicate by adding the following volumes to 100 μL of blank matrix (whole blood, serum or plasma): 100 μL of the standards, 100 μL of the internal standards mix solution and 200 μL of methanol (methanol total volume 400 μL). The standards were vortexed for 30 s and centrifuged for 10 min at 10,000 rpm. An aliquot of 200 μL of the supernatant was transferred to LC vial and diluted with 1.3 mL of deionized water. A 5 μL aliquot of the diluted supernatant was injected and analyzed by LC–MS-MS.

Specimens

Human blank whole blood, plasma and serum were obtained from Golden West Biological, Inc.® (CA, USA). Postmortem blood ($n = 5$) and serum samples ($n = 25$) were kindly provided by NMS Lab (PA, USA) collected over 2 months and stored in the freezer at −20°C.

Sample preparation

A 100 μL aliquot of the sample (blood, plasma or serum) was transferred to a 2 mL snap top polypropylene microcentrifuge tube. To this, 100 μL of combined internal standard solution and 300 μL of methanol were added (methanol total volume 400 μL). The samples were vortexed for 30 s and centrifuged for 10 min at 10,000 rpm. An aliquot of 200 μL of the supernatant was transferred to an LC vial and diluted with 1.3 mL of demonized water. A 5 μL aliquot of the diluted supernatant was injected and analyzed by LC–MS-MS.

Instrumentation

An Agilent LC–MS-MS triple quadruple G6430A mass spectrometer equipped with Agilent 1200 series autosampler, quaternary pump SL with degasser and thermostatted column compartment was used. Electrospray ionization was used and the MS operated in multiple reaction monitoring mode with ion mode switching. The optimal conditions were achieved using a nebulizer pressure at 15 psi, a capillary voltage of 4,000 V, nitrogen gas heated to 300°C and delivered at 10 mL/min. The column used was a Phenomenex Gemini C18 (150 × 2.1 mm, 5 μm) coupled with a C18 guard column (4 × 2.0 mm). The column temperature was maintained at 40°C. Gradient elution was employed using a mobile phase consisting of A: 2 mM ammonium acetate in water and B: 2 mM ammonium acetate in methanol at a flow rate of 0.3 mL/min. The total run time was 17 min. The gradient mobile phase system started at 80 : 20 A/B increasing to 50 : 50 A/B within 2 min. This percentage was maintained for 6 min before being increased to 10 : 90 A/B for 2 min. The percentage was finally decreased to 80 : 20 A/B for 7 min in order to condition the column before the next injection. Data analysis were performed using Agilent MassHunter Workstation (version: B.01.05).

Method validation

The method has been validated according to the standard practices for method validation in forensic toxicology (SWGTOX, May 2013) for whole blood, serum and plasma (32).

Selectivity was evaluated in blood by comparing the chromatograms of 11 different blank sources (donors) with those of corresponding standards spiked with 22 AEDs. Selectivity in plasma/serum was evaluated using negative case samples.

Specificity was assessed by spiking drug-free matrix with each AED individually.

Linearity was assessed by analyzing five separate calibration curves per matrix prepared by spiking blank blood, plasma or serum with AEDs working solutions at eight concentrations ranging from 5 to 300 μg/mL for VIG, VPA and LEV; 0.05 to 10 μg/mL for TIG and RTG; 0.1 to 10 μg/mL for NA-RTG and OXC; 0.5 to 50 μg/mL for CBZ, CBZ0, ESL, LIC, GBP, LAC, RFM, STP and TPR; 1–50 μg/mL LTG, PHT, *p*-HPPH, PGR and ZNS; and 2.5–50 μg/mL for PBT. All calibrations were prepared freshly in duplicate over five different days. Calibration curves were generated by plotting the peak area ratio versus the spiked analyte concentrations using the simplest least-squares linear regression model and Agilent MassHunter Workstation-Quantitative software. Blank matrix with internal standard was run with each batch but not included in the calibration curve. The correlation of coefficient (R^2) was calculated. The R^2 values should be >0.99.

Limits of detection (LOD): Instrument LODs were determined using decreasing concentrations of unextracted drugs standards

solutions and analyzing in duplicate for three separate runs using three different matrix sources. Assay LODs were determined for each matrix (blood, plasma and serum) using blank matrix samples spiked with decreasing concentrations of AEDs and also analyzed in duplicate for three separate runs. Instrument and assay LOD are considered the lowest concentration that gives a reproducible instrument response with signal-to-noise (S/N) ratio ≥ 3.

Lower limit of quantification (LLOQ) is considered the lowest concentration that gives a reproducible instrument response with a co-efficient of variation (CV%) $<20\%$ and S/N ≥ 10. MassHunter station was used to calculate the S/N ratio.

Limit of quantification (LOQ) was determined on the basis of the clinical need and has a CV% $<20\%$ and S/N ≥ 10.

Bias and precision were assessed by analyzing replicates of spiked controls at three different concentrations (low, medium and high).

Intraday precision was calculated from 3 to 6 replicates per QC per matrix in one batch.

Interday precision was determined over five different runs.

Bias was expressed as percentage of the nominal concentration

Precision was established by the percentage of the CV%.

A stability study was carried on using drug-free matrix (blood, plasma and serum) spiked in triplicate with the 22 AEDs at three different concentrations (low, medium and high). These spiked samples were initially analyzed to establish time zero concentrations. Storage conditions evaluated were room temperature ($\sim 20^{\circ}C$) for 24 h, in process stability (autosampler) and three freeze–thaw cycles at $-20^{\circ}C$. The concentrations obtained were compared with the average time zero concentrations. Autosampler stability ($\sim 20^{\circ}C$) was evaluated by re-injecting QCs at three concentrations after 24, 48 and 72 h. The analyte was considered stable if the concentration compared with the time zero concentration exceeded $\pm 10\%$.

Recoveries and matrix effects were evaluated for all the drugs and the internal standards using the postextraction addition approach (33). Eleven different blank matrix sources (donors) were spiked with either low or high QC and analyzed in triplicate.

Matrix effect was represented by the matrix factor (MF), which is defined as the ratio of peak area of standards added to extracted blank matrix (postextraction spike) to that of standards solutions at the same concentrations (pure standards). If MF is equal to 1 this means that no matrix effects are present. MF <1 means there is ionization suppression, whereas MF >1 may be due to ionization enhancement and/or analyte loss in the absence of matrix.

Recovery was calculated by dividing the peak area of extracted standards (pre-extraction spike) by the peak area of the extracted pooled matrix spiked with standards after extraction at the same concentration (postextraction spike).

Carryover was tested by injecting three blank controls after the highest concentration in the calibration curve.

Case studies

To prove the applicability of this method, the method was verified with an anonymous set of 5 postmortem blood and 25 serum samples that had previously been quantified by NMS Labs using a variety of different analytical techniques (GC–MS, HPLC and LC–MS-MS).

Results and discussion

AEDs separation and chromatographic condition optimization

The mass spectrometry parameters were optimized using the infusion of the 22 compounds prepared in the mobile phase in individual solutions. Optimized results for precursor ions, product ions, fragmentor voltage and collision energies are summarized in Table I.

ESL is a chiral pro-drug, which is quickly and extensively metabolized to *S*-LC (95–98%) and, to a minor extent, *R*-licarbazepine (*R*-LC) and OXC (34). On the other hand, OXC is an achiral pro-drug which, in humans, is reduced to the active licarbazepine metabolite, appearing in plasma as *S*-LC and *R*-LC in an $\sim 4:1$ enantiomer ratio (35). As a result, the method has been validated using *S*-LC as a metabolite of both OXC and ESL. The chromatograms of 22 AEDs are shown in Figure 1.

Method validation results

The method has been developed and validated for 22 AEDs and/or metabolites including acidic, basic and amphoteric compounds with a wide range of pK_a and different polarities as indicated in Figure 2.

Specificity and selectivity

The specificity and selectivity of the method were investigated by analyzing 11 different sources of matrix. No endogenous interference was observed in drug-free matrix at the retention times of the target drugs. None of the AEDs or the internal standards showed any interference at the peak area of the other drugs included in the method.

Table I
Optimization of MRM Transitions, Fragmentor Voltage, Collision Energy, Ionization Mode and Retention Time of 22 AEDs

AEDs	Precursor (*m/z*)	Quantifier (*m/z*)	Qualifier (*m/z*)	Frag[a] (V)	CE[b] (eV)	RT[c] (minutes)	Ionization mode
CBZ	237.3	194.2	192.0	140	20	12.0	+
CBZD	253.1	236.1	210.1	90	2	8.7	+
ESL	297.2	194.1	237.2	100	20	11.2	+
GBP	172.2	154.2	137.0	110	10	3.4	+
LAC	251.1	108.1	91.1	90	5	7.3	+
LEV	171.1	154.0	69.1	50	5	3.7	+
S-LC	255.1	194.1	237.2	80	20	8.6	+
LTG	256.1	166.0	211.0	180	30	7.8	+
OXC	253.2	180.1	208.1	170	30	9.4	+
PBT	231.1	188.2	85.0	100	5	8.3	–
PGB	160.2	142.2	97.1	140	10	3.4	+
PHT	251.2	102.0	208.2	110	10	11.0	–
p-HPPH	267.2	118.1	224.1	125	10	7.9	–
RFM	239.0	127.0	222.0	90	20	7.3	+
RTG	304.2	230.1	258.1	140	20	12.9	+
NA-RTG	274.3	256.0	232.1	115	10	9.3	+
STP	217.2	159.2	187.2	100	10	14.3	+
TIG	376.1	247.1	278.2	140	20	13.4	+
TPR	338.1	78.0	96.0	160	20	8.6	–
VIG	130.1	113.1	71.0	65	10	1.6	+
VPA	143.1	143.1	n/a	100	0	11.7	–
ZNS	211.2	119.1	147.1	115	10	6.5	–
Internal standards							
CBZ-DiOH	239.1	194.1	n/a	140	20	12.8	+
GBP-D_{10}	182.1	164.3	n/a	110	10	3.3	+
TUB	269.2	170.0	n/a	120	10	11.1	–

[a]Fragmentor voltage.
[b]Collision energy.
[c]Retention time.

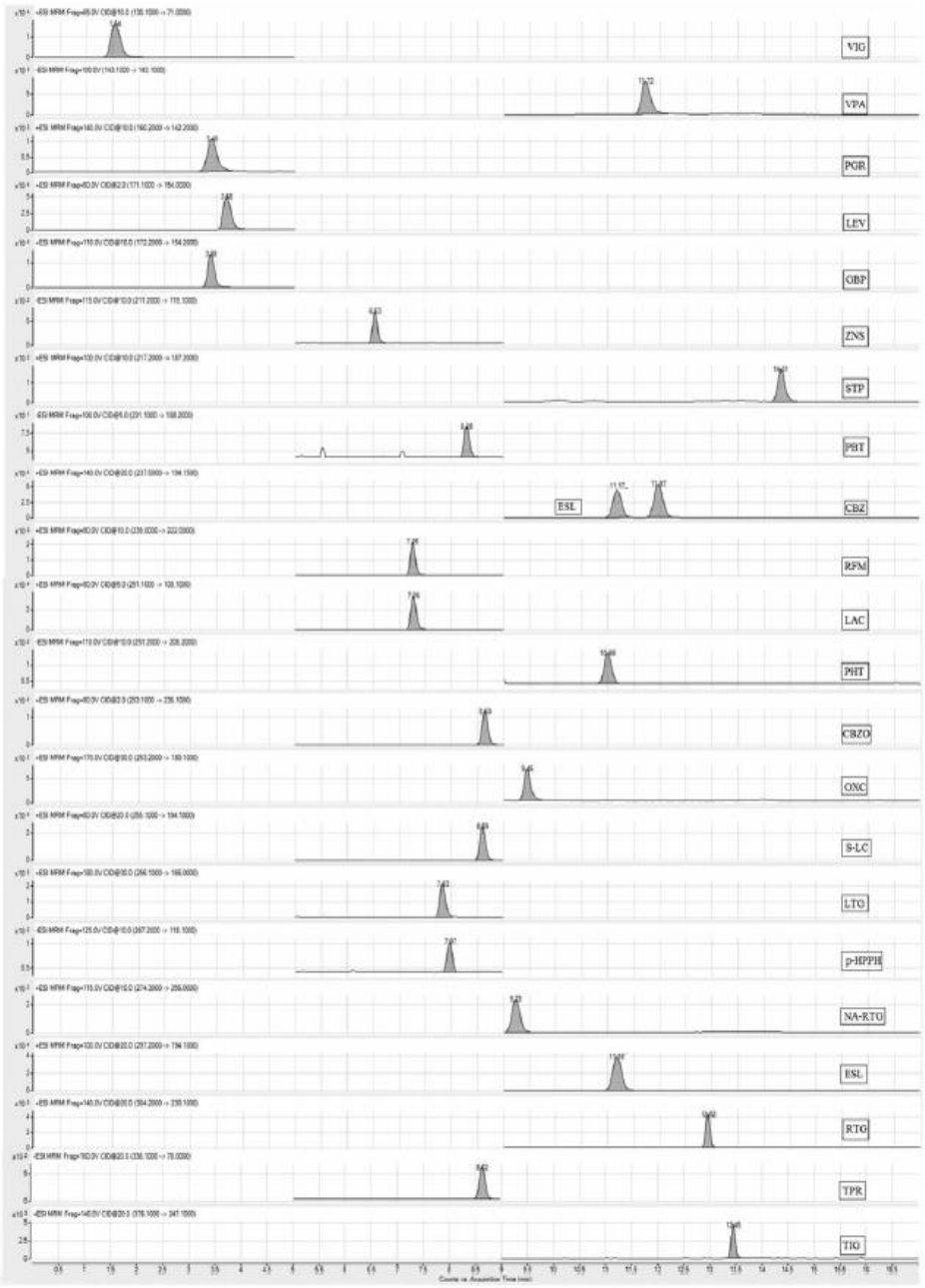

Figure 1. The chromatogram of the 22 AEDs using the extracted medium QC from plasma.

Linearity, LOD, LLQ and LOQ

The same calibration models were used for blood, serum and plasma. They were linear over the wide range of concentrations tested with an $R^2 > 0.99$ using a weighting of $1/X$ for all drugs in whole blood, serum and plasma except for CBZ, CBZO, ESL, LAC, LEV, LTG and VIG where quadratic regressions were used with a weighting of $1/X$. Assay LODs, LLOQs and LOQs had the same values in blood, plasma and serum, whereas the instrument LODs were slightly lower than the assay LODs for ESL, S-LC, NA-RTG and VIG (Table II).

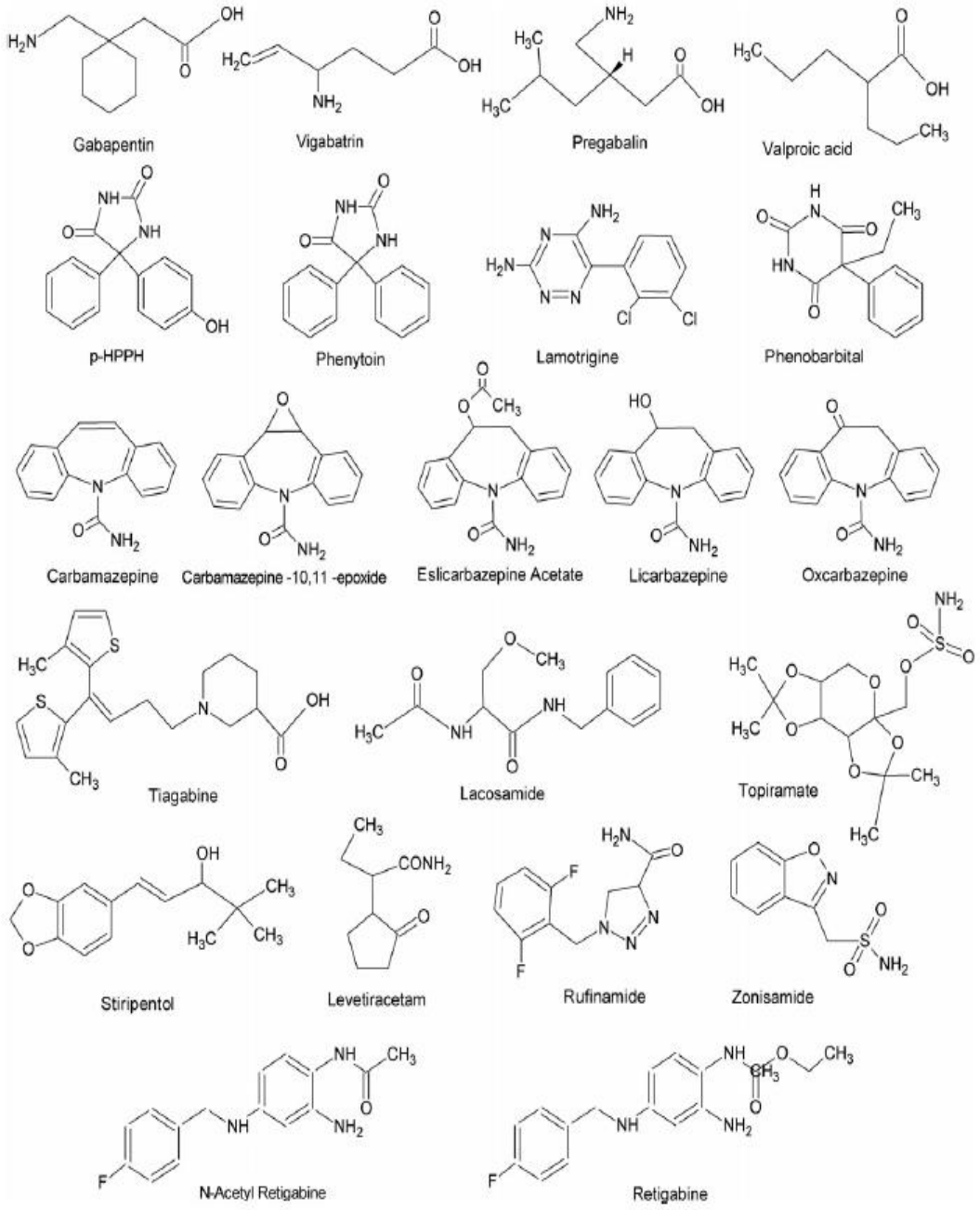

Figure 2. The chemical structure of 22 AEDs.

Accuracy and precision

Accuracy and precision results are presented in Tables III–V. The intraday precision values were <9% for whole blood, <10% for plasma and <8.7% for serum. The interday precision values were <12.1% for whole blood, <13.1% for plasma and serum (except PBT where the medium QC was 16.2% in plasma). The accuracy values were within the acceptable range of ±15% of the nominal concentrations. The inter- and intraday accuracy was between 92.7 and 108.7% for whole blood, between 89.6 and 110.6% for plasma and between 92.1 and112.5% for serum.

MF, recovery and carryover

Table VI summarizes the MF and recovery results of two QCs (low and high) using 11 different sources of matrix (data for internal standards are not shown). MF values were within the acceptable range for all the drugs and the internal standards

Table II
Calibration Model, LOD, LLOQ, LOQ and Linearity Results in Whole Blood, Plasma and Serum

AEDs	Theraputic level (mg/L)	Calibration range (mg/L)	Calibration model	Internal standards	Instrument LOD	Assay LOD	LLOQ	LOQ	Whole blood R^2 ($n = 5$)	Plasma R^2 ($n = 5$)	Serum R^2 ($n = 5$)
CBZ	1.7–15.0	0.5–50.0	Quadratic	CBZ-DiOH	0.1	0.25	0.5	0.5	0.999	0.999	0.999
CBZD	0.5–2.0	0.5–50.0	Quadratic	CBZ-DiOH	0.05	0.05	0.25	0.5	0.999	0.998	0.998
ESL	10.0–26.0	0.5–50.0	Quadratic	CBZ-DiOH	0.1	0.25	0.5	0.5	0.999	0.996	0.999
GBP	5.0–9.0	0.5–50.0	Linear	GBP-D_{10}	0.1	0.1	0.25	0.5	0.998	0.998	0.999
LAC	2.5–14.0	0.5–50.0	Quadratic	GBP-D_{10}	0.05	0.05	0.25	0.5	0.999	0.997	0.998
LEV	10.0–40.0	5.0–300.0	Quadratic	GBP-D_{10}	0.1	0.1	0.5	5.0	0.999	0.999	0.999
S-LC	6.0–25.0	0.5–50.0	Linear	CBZ-DiOH	0.05	0.1	0.25	0.5	0.9997	0.999	0.998
LTG	2.3–5.6	1.0–50.0	Quadratic	GBP-D_{10}	0.25	0.25	0.5	0.5	0.9995	0.997	0.998
OXC	0.05–1.2	0.1–10.0	Linear	CBZ-DiOH	0.05	0.05	0.1	0.1	0.999	0.999	0.999
PBT	10.0–40.0	2.5–50.0	Linear	TUB	1.0	1.0	2.5	2.5	0.999	0.995	0.998
PGB	1.0–5.0	1.0–50.0	Linear	GBP-D_{10}	0.5	0.5	1.0	1.0	0.997	0.999	0.998
PHT	7.0–20.0	1.0–50.0	Linear	TUB	0.5	0.5	1.0	1.0	0.9997	0.996	0.998
p-HPPH	1.0–40.0	1.0–50.0	Linear	TUB	0.5	0.5	1.0	1.0	0.998	0.999	0.999
RFM	2.0–7.0	0.5–50.0	Linear	GBP-D_{10}	0.1	0.1	0.25	0.5	0.999	0.996	0.996
RTG	0.5–1.9	0.05–10.0	Linear	CBZ-DiOH	0.025	0.025	0.05	0.05	0.999	0.998	0.997
NA-RTG	0.01–0.2	0.1–10.0	Linear	CBZ-DiOH	0.01	0.025	0.05	0.1	0.996	0.996	0.997
STP	4.0–22.0	0.5–50.0	Linear	TUB	0.25	0.25	0.5	0.5	0.994	0.998	0.998
TIG	0.03–1.0	0.05–10.0	Linear	CBZ-DiOH	0.01	0.01	0.025	0.05	0.998	0.997	0.997
TPR	2.4–27.0	0.5–50.0	Linear	TUB	0.25	0.25	0.5	0.5	0.997	0.998	0.999
VIG	18.0–77.0	5.0–300.0	Quadratic	GBP-D_{10}	0.1	0.5	1.0	5.0	0.999	0.998	0.999
VPA	50.0–100.0	5.0–300.0	Linear	TUB	2.5	2.5	5.0	5.0	0.997	0.999	0.999
ZNS	1.0–50.0	1.0–50.0	Linear	TUB	0.5	0.5	1.0	1.0	0.996	0.999	0.999

Table III
Intra- and Interday Precision and Accuracy Results of Whole Blood

AEDs	Precision						Accuracy					
	Intraday (%)			Interday (%)			Intraday (%)			Interday (%)		
Whole blood	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
CBZ	2.6*	2.8*	1.6*	9.2*	3.8*	4.6*	100.8*	99.2*	96.8*	100.8*	99.2*	96.8*
CBZD	4.9*	2.3*	1.5*	4.3*	3.6*	4.0*	95.9*	101.5*	102.5*	95.8*	101.5*	102.5*
ESL	2.1*	2.6*	1.6*	3.5*	6.9*	2.9*	97.5*	101.9*	98.8*	97.5*	101.9*	98.8*
GBP	4.3*	2.4*	2.9*	3.1*	4.3*	4.1*	99.9*	102.7*	100.6*	102.4*	102.7*	100.6*
LAC	4.4*	3.1*	3.6*	11.0*	6.3*	5.5*	92.7*	102.7*	99.7*	92.7*	102.7*	99.7*
LEV	3.1*	3.5*	4.1*	12.1*	6.9*	4.5*	95.1*	99.3*	101.3*	95.1*	99.3*	101.3*
S-LC	2.2	4.8	6.6	5.8	5.4	7.4	105.9	108.4	101.3	105.9	108.4	101.3
LTG	7.8*	4.3*	4.8*	11.1*	9.7*	11.6*	101.9*	95.9*	100.3*	101.9*	103.3*	100.3*
OXC	2.6*	2.0*	2.0*	4.4*	2.0*	3.0*	102.7*	97.5*	103.1*	102.7*	97.5*	103.1*
PBT	4.1	5.9	9.0	2.9	12.9	5.2	98.8	98.3	103.0	94.9	98.3	103.0
PGB	4.2*	2.3*	2.5*	5.4*	2.6*	3.0*	98.3*	101.4*	102.1*	98.3*	101.4*	102.1*
PHT	6.1*	1.3*	0.7*	7.6*	2.0*	0.3*	101.7*	98.2*	101.1*	101.7*	98.2*	101.1*
p-HPPH	8.4*	2.8*	2.2*	8.1*	8.5*	4.3*	101.8*	99.3*	100.7*	101.8*	99.4*	100.7*
RFM	2.4	4.1	3.2	3.7	9.7	3.5	108.7	106.0	96.4	108.7	106.0	96.4
RTG	6.5	6.4	6.6	4.7	6.7	8.0	101.8	106.8	106.1	101.8	106.8	106.1
NA-RTG	7.8	5.5	5.7	12.7	8.8	7.0	98.5	100.5	101.7	98.5	100.5	101.7
STP	5.9	6.2	7.1	4.6	9.6	3.5	106.5	98.7	101.4	106.5	98.7	101.5
TIG	5.7*	2.4*	2.0*	6.8*	4.8*	3.9*	100.6*	100.1*	97.5*	100.6*	100.1*	97.5*
TPR	2.7*	1.7*	1.7*	5.5*	4.0*	4.7*	98.0*	103.4*	102.0*	98.0*	103.4*	102.0*
VIG	5.7	4.3*	2.8*	10.4	5.8*	4.0*	98.7	101.5*	102.1*	98.7	101.5*	102.1*
VPA	1.2*	0.7*	0.4*	4.8*	0.3*	0.4*	100.4*	100.5*	100.5*	100.4*	100.5*	100.5*
ZNS	5.9*	3.7*	2.7*	13.0*	3.7*	4.0*	94.3*	98.2*	100.3*	94.3*	98.2*	100.3*

$n = 15$ except (*) indicates $n = 30$.

(within ± 1.25) with SD $< 20\%$. Recovery was $> 79\%$ for all the AEDs and the internal standards in the three matrices except RTG and its metabolite NA-RTG, which were $\sim 70\%$ in whole blood compared with their recoveries from serum and plasma, which were $> 93\%$. No carry over was observed in the blank samples after two injection of the highest standards.

Stability

During the validation process, the stability study showed that all the drugs were stable in the whole blood, serum and plasma at room temperature ($\sim 20^{\circ}C$) for up to 24 h (bench top stability) except RTG, NA-RTG, ESL and OXC, which showed a loss of 40% of their nominal concentrations in the three matrices. All AEDs including RTG, NA-RTG, ESL and OXC were stable after three freeze–thaw cycles at $-20^{\circ}C$ and the extracted samples were stable in the autosampler ($\sim 20^{\circ}C$) for up to 72 h. The results showed that RTG, NA-RTG, ESL and OXC are not stable in whole blood but their stability was acceptable after their extraction and reconstitution in 13% methanol in water.

Case studies

The method was verified with a set of 30 samples (5 postmortem blood samples, 25 serum/plasma samples) that had previously been quantitated during routine case analysis.

Table IV
Intra- and Interday Precision and Accuracy Results of Plasma

AEDs	Precision						Accuracy					
	Intraday (%)			Interday (%)			Intraday (%)			Interday (%)		
Plasma	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
CBZ	1.1	2.2	1.6	1.0	0.2	4.0	97.9	101.3	102.5	97.9	101.3	102.5
CBZO	3.2	0.9	1.9	4.9	3.3	1.6	101.0	104.6	101.1	101.0	104.6	101.1
ESL	1.8	4.9	1.8	2.4	4.3	5.4	99.9	93.4	99.5	99.9	106.7	99.5
GBP	1.4	2.6	0.8	0.8	2.7	3.5	96.2	98.3	104.6	105.8	98.3	104.6
LAC	1.5	1.7	1.3	1.9	3.6	4.5	99.2	100.1	100.7	99.2	100.1	100.7
LEV	2.6	1.4	3.6	2.1	4.2	2.9	96.5	96.3	97.3	96.5	96.3	97.3
S-LC	1.4	1.3	4.3	5.4	5.6	0.7	101.1	101.5	91.7	101.1	101.5	91.7
LTG	2.8	2.6	3.1	6.5	10.3	10.7	110.6	103.3	99.9	110.6	103.3	103.1
OXC	2.3	4.3	2.7	4.6	1.6	3.7	95.2	107.7	100.2	95.2	107.7	100.2
PBT	5.0	16.2	6.0	7.9	4.7	3.9	93.7	92.2	105.0	93.7	92.2	91.9
PGR	10.0	6.1	4.6	2.0	3.9	3.8	96.6	103.8	101.8	106.3	103.8	101.8
PHT	8.7	3.6	2.0	4.3	12.0	12.0	102.1	98.0	105.0	102.1	98.0	107.0
p-HPPH	9.9	7.4	4.5	2.4	10.9	2.8	95.4	100.2	97.9	95.4	100.2	97.9
RFM	3.3	1.3	0.9	13.1	4.2	0.2	107.1	104.0	102.5	107.1	104.0	102.5
RTG	2.6	6.9	2.1	0.8	6.5	8.2	89.6	106.5	96.2	99.1	106.5	100.0
NA-RTG	2.3	2.6	2.7	2.8	4.7	4.8	107.8	103.4	102.8	107.8	103.4	102.8
STP	5.0	3.8	3.2	2.8	9.9	6.0	98.5	108.7	103.8	103.8	100.1	98.5
TIG	3.2	2.2	1.0	9.1	8.0	9.2	102.5	108.8	96.1	102.5	102.9	96.6
TPR	7.6	3.7	3.3	7.6	2.0	1.5	103.9	103.3	103.1	103.9	103.3	103.1
VIG	2.6	4.9	1.4	5.6	2.6	1.7	98.5	97.3	98.9	98.5	97.3	98.9
VPA	3.5	3.0	2.7	4.3	3.9	0.7	95.0	101.4	104.0	95.0	101.4	104
ZNS	8.7	7.2	3.1	7.3	8.6	3.9	105.6	98.9	108.5	105.6	98.9	95.0

Table V
Intra- and Interday Precision and Accuracy Results of Serum

AEDs	Precision						Accuracy					
	Intraday (%)			Interday (%)			Intraday (%)			Interday (%)		
Serum	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
CBZ	2.4	1.9	1.1	1.0	0.7	2.6	98.2	98.3	99.9	98.2	98.3	99.9
CBZO	2.8	0.9	2.9	4.8	0.4	0.5	94.1	103.6	95.4	94.1	103.6	95.4
ESL	2.2	3.6	1.9	5.4	5.0	7.7	93.2	85.7	96.8	93.2	97.9	96.8
GBP	2.2	2.5	2.0	1.8	3.9	0.6	96.7	96.3	103.3	106.3	96.3	103.3
LAC	1.9	2.7	3.7	4.2	1.1	2.7	96.2	96.3	100.7	96.2	96.3	100.7
LEV	1.8	2.2	0.8	0.6	0.9	3.7	94.3	96.7	94.2	94.3	96.7	94.2
S-LC	1.1	1.8	2.3	1.7	2.0	2.1	106.2	107.3	104.8	106.2	107.3	104.8
LTG	1.2	1.6	2.3	3.7	2.6	4.8	107.9	94.6	92.1	107.9	94.6	92.1
OXC	2.0	2.7	2.8	4.6	1.0	4.7	95.3	102.9	97.8	95.3	102.9	97.8
PBT	3.1	8.0	4.3	4.0	10.0	2.8	107.7	102.0	97.4	107.0	101.0	97.4
PGR	4.6	9.0	3.3	5.5	9.6	4.9	105.9	101.9	97.9	105.9	101.9	97.9
PHT	5.5	3.1	2.9	2.2	4.3	5.7	94.3	101.5	113.2	94.3	96.9	109.8
p-HPPH	1.6	8.7	4.2	7.7	5.7	5.7	101.4	102.8	99.4	101.4	102.8	99.4
RFM	1.8	3.0	1.7	7.2	1.2	3.4	105.3	107.7	102.1	105.3	107.7	102.1
RTG	2.1	1.3	2.6	2.2	0.5	7.6	103.0	110.5	97.6	103.0	110.5	97.6
NA-RTG	1.9	2.5	1.6	4.5	1.6	3.8	107.5	98.8	100.2	107.5	98.8	100.2
STP	1.2	4.4	2.9	6.8	7.6	2.9	104.1	98.6	106.4	104.1	110.9	106.4
TIG	1.6	0.7	4.3	7.7	6.2	5.7	111.8	92.3	103.2	111.8	92.3	91.8
TPR	4.0	4.6	4.7	3.3	4.0	3.3	106.0	102.1	100.8	106.0	102.1	100.8
VIG	1.2	3.1	1.9	2.0	6.6	7.8	93.0	93.8	96.8	93.0	93.8	96.8
VPA	4.8	4.6	4.3	0.6	3.2	2.4	94.0	102.7	107.6	94.0	102.7	107.6
ZNS	3.1	3.6	6.1	2.0	13.1	5.3	101.8	104.2	112.5	101.8	104.2	98.4

Figure 3 shows the correlation of comparison for the quantitation results with the reference lab methods for 28 of the 30 samples. The correlation coefficient was very good giving an R^2 of 0.982. No interferences were observed with any of the case samples. Cases 17 and 29 are not represented in Figure 3 (PBT: 42.3 mg/L, ZNS: 40.6 mg/L, respectively) to avoid skewing the data. Although their concentrations were within the therapeutic ranges, they are much higher than the concentrations for the drugs in the other cases, which may produce a biased R^2 value. The two results obtained for these cases are in good agreement with the original labs results.

Table VII summarizes that more than half of the samples tested had 2–4 AEDs present. PHT was detected in six cases and the concentration ranged between 3.1 and 16.4 mg/L. The individuals in these cases had been prescribed fosphenytoin. Fosphenytoin is a phosphate ester pro-drug of PHT, which does not have significant pharmacological activity (36). Therefore, the clinical monitoring is concerned only with the plasma concentrations of the derived PHT.

Table VI
Recovery and Matrix Factor Values for 22 AEDs Using Low and High QCs and 11 Different Matrix Sources ($n = 6$ per QC per Matrix)

AEDs	Whole blood				Plasma				Serum			
	QC1		QC3		QC1		QC3		QC1		QC3	
	Recovery (%)	Matrix factor	Recovery (%)	Matrix factor	Recovery (%)	Matrix factor	Recovery (%)	Matrix factor	Recovery (%)	Matrix factor	Recovery (%)	Matrix factor
CBZ	106 ± 3.0	1.05 ± 0.07	104 ± 3.1	1.0 ± 0.02	102 ± 2.4	1.06 ± 0.01	98 ± 1.4	1.10 ± 0.04	104 ± 1.4	1.04 ± 0.01	103 ± 2.7	1.04 ± 0.08
CBZO	105 ± 3.2	1.05 ± 0.05	104 ± 1.6	1.01 ± 0.02	99 ± 2.0	1.07 ± 0.02	99 ± 1.5	1.08 ± 0.04	103 ± 0.3	1.04 ± 0.02	102 ± 3.0	1.03 ± 0.05
ESL	107 ± 2.7	1.07 ± 0.05	106 ± 1.9	1.04 ± 0.04	99 ± 2.7	1.04 ± 0.02	95 ± 5.8	1.06 ± 0.05	104 ± 3.2	1.0 ± 0.02	102 ± 5.2	0.99 ± 0.06
GBP	109 ± 3.6	0.99 ± 0.08	106 ± 2.6	1.01 ± 0.03	100 ± 3.2	1.04 ± 0.02	98 ± 0.9	1.13 ± 0.06	106 ± 1.4	0.99 ± 0.02	103 ± 4.6	1.03 ± 0.06
LAC	108 ± 2.5	1.06 ± 0.07	107 ± 2.3	1.0 ± 0.02	104 ± 2.4	1.09 ± 0.01	99 ± 1.8	1.10 ± 0.03	104 ± 0.3	1.07 ± 0.01	103 ± 2.8	1.04 ± 0.05
LEV	109 ± 4.0	1.08 ± 0.06	109 ± 5.9	0.97 ± 0.02	101 ± 2.9	1.13 ± 0.03	97 ± 2.0	1.06 ± 0.04	105 ± 1.3	1.10 ± 0.01	105 ± 4.9	0.99 ± 0.08
S-LC	108 ± 3.1	1.04 ± 0.06	106 ± 2.9	0.99 ± 0.01	105 ± 1.4	1.11 ± 0.01	102 ± 2.1	1.12 ± 0.03	104 ± 0.8	1.09 ± 0.01	105 ± 6.1	1.09 ± 0.08
LTG	101 ± 22.7	1.14 ± 0.22	98 ± 12.6	1.08 ± 0.16	86 ± 4.0	1.2 ± 0.04	105 ± 19.1	1.14 ± 0.2	102 ± 3.9	0.98 ± 0.05	104 ± 2.7	1.16 ± 0.09
OXC	110 ± 6.5	1.0 ± 0.07	108 ± 7.3	0.91 ± 0.03	109 ± 6.6	1.03 ± 0.06	96 ± 2.2	1.03 ± 0.05	105 ± 1.5	1.0 ± 0.06	110 ± 7.6	0.91 ± 0.06
PBT	108 ± 24.2	0.93 ± 0.24	107 ± 17.2	1 ± 0.10	79 ± 20.9	1.24 ± 0.17	99 ± 5.7	1.05 ± 0.07	98 ± 14.4	1.07 ± 0.19	108 ± 11.7	0.96 ± 0.01
PGR	100 ± 8.2	1.11 ± 0.08	107 ± 4.8	1.04 ± 0.03	100 ± 6.6	1.05 ± 0.08	97 ± 2.4	1.15 ± 0.06	109 ± 4.6	1.04 ± 0.05	104 ± 5.8	1.06 ± 0.06
PHT	95 ± 9.2	1.09 ± 0.08	106 ± 7.1	0.99 ± 0.05	95 ± 12.6	1.10 ± 0.14	98 ± 4.4	1.06 ± 0.08	104 ± 11.0	1.02 ± 0.07	105 ± 6.4	1.01 ± 0.06
p-HPPH	96 ± 7.1	1.04 ± 0.09	106 ± 9.7	0.97 ± 0.06	109 ± 9.9	1.07 ± 0.04	99 ± 13.4	1.04 ± 0.08	109 ± 15.1	1.06 ± 0.05	102 ± 8.5	0.97 ± 0.08
RFM	106 ± 3.8	1.07 ± 0.06	107 ± 3.1	1.0 ± 0.03	102 ± 2.5	1.07 ± 0.04	98 ± 2.2	1.09 ± 0.06	104 ± 2.2	1.06 ± 0.01	103 ± 4.1	1.03 ± 0.07
RTG	73 ± 9.1	0.85 ± 0.05	71 ± 2.6	0.83 ± 0.04	102 ± 4.5	0.88 ± 0.02	93 ± 6.8	0.94 ± 0.05	107 ± 12.5	0.96 ± 0.03	112 ± 12.1	0.89 ± 0.07
NA-RTG	66 ± 2.5	0.89 ± 0.06	68 ± 3.9	0.88 ± 0.02	103 ± 1.7	0.99 ± 0.03	100 ± 2.7	1.07 ± 0.05	106 ± 3.5	0.98 ± 0.01	103 ± 5.1	1.02 ± 0.08
STP	109 ± 6.3	1.10 ± 0.06	109 ± 3.2	1.0 ± 0.03	99 ± 4.5	1.15 ± 0.03	99 ± 1.0	1.06 ± 0.05	102 ± 5.3	1.15 ± 0.05	105 ± 6.1	1.02 ± 0.09
TIG	103 ± 18.2	0.93 ± 0.10	101 ± 8.2	1.01 ± 0.02	94 ± 22.9	0.93 ± 0.17	102 ± 7.1	1.09 ± 0.11	108 ± 6.9	0.98 ± 0.10	103 ± 3.7	1.04 ± 0.05
TPR	107 ± 5.0	1.10 ± 0.07	108 ± 6.3	1.0 ± 0.03	98 ± 6.4	1.18 ± 0.03	98 ± 1.3	1.07 ± 0.05	105 ± 3.6	1.14 ± 0.04	107 ± 6.5	0.98 ± 0.07
VIG	105 ± 3.3	0.75 ± 0.07	111 ± 7.3	0.83 ± 0.02	98 ± 1.9	1.11 ± 0.03	93 ± 3.0	1.12 ± 0.06	102 ± 2.7	0.77 ± 0.01	101 ± 4.2	0.83 ± 0.06
VPA	106 ± 8.4	1.05 ± 0.10	108 ± 4.4	1.0 ± 0.04	100 ± 6.0	1.06 ± 0.07	102 ± 2.8	1.04 ± 0.05	103 ± 4.1	1.07 ± 0.12	108 ± 6.6	0.98 ± 0.08
ZNS	107 ± 2.5	1.05 ± 0.08	105 ± 4.7	0.99 ± 0.02	104 ± 3.9	1.08 ± 0.13	98 ± 2.6	1.06 ± 0.05	108 ± 3.7	1.08 ± 0.14	107 ± 6.0	1.0 ± 0.08

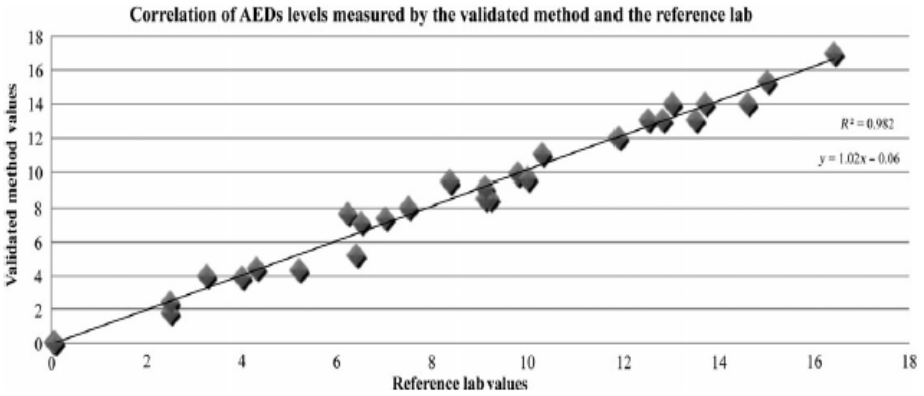

Figure 3. Correlation of AEDs concentrations levels measured with the validated method versus the values obtained by NMS Lab, for 28 samples.

All AED concentrations measured were within therapeutic ranges except the PGR in cases 18 and 19 where the concentrations were slightly higher than the therapeutic range (12.5 and 14.6 mg/L, respectively). GBP concentrations were higher than the therapeutic range (37) in two cases (19 and 24) but GBP analysis was not requested for either case. Case 19 has PGR and GBP and they are both higher than the therapeutic levels required for epilepsy maintenance; however, no data are available regarding the concentrations found in patients taking these drugs for neuropathic pain. As no case history is available, it is not possible to comment if these patients are abusing these drugs or being medicated for something other than epilepsy.

There was one case (Case 25) where OXC and *S*-LE were detected with concentrations of <0.05 and 13.9 mg/L, respectively, but ESL analysis gave negative results. As this case was tested for TIG only, it was not possible to determine whether the compounds found are OXC and its metabolite *S*-LC or if they are ESL with its two metabolites OXC and S-LC.

In general, clinical and forensic labs analyze AEDs only when requested by clinicians or pathologists and usually the drugs are specified, although ~20% of epileptic patients are using a polytherapy of AEDs (38). For example, four cases have been tested for only one drug, although the sample had another three drugs detected by the validated method. The analysis cost and time in addition to the ability to afford state of art techniques can be obstacles, which may affect the number of tests requested. Therefore, the development of the simultaneous analysis of AEDs using a small sample volume and a simple extraction procedure may improve the TDM of these drugs and can play an important role in enhancing the quality of life for epileptic people. With increased awareness of poly-AED use, the incidence of SUDEP could be reduced. Finally, by monitoring all the medications

Table VII
AEDs Levels Measured by the Validated Method and the Reference Lab

Case No.	Matrix	No. of AEDs	Requested AEDs	Method conc. (mg/L)	Ref lab conc. (mg/L)	Ref lab method	Other AEDs tested (mg/L)
Case 1	Postmortem blood	1	GBP	8.4	9.5	LC–MS-MS	–
Case 2	Postmortem blood	1	LTG	6.2	7.6	HPLC	–
Case 3	Postmortem blood	1	PHT	3.3	4.0	HPLC	–
Case 4	Postmortem blood	1	PHT	13.7	14.0	HPLC	–
Case 5	Postmortem blood	1	PHT	16.4	17.0	HPLC	–
Case 6	Serum	1	CBZ	13.5	13	HPLC	CBZO (2.9)
Case 7	Serum	4	CBZ	9.2	8.5	HPLC	TPR (7.4), LTG (8.2), LEV (43.0)
	Serum		CBZO	2.5	1.8	HPLC	
Case 8	Serum	1	GBP	9.8	9.9	LC–MS-MS	–
Case 9	Serum	2	GBP	13.0	14.0	LC–MS-MS	CBZ (14.0), CBZO (3.4)
Case 10	Serum	1	GBP	4.3	4.4	LC–MS-MS	–
Case 11	Serum	3	LAC	7.5	8.0	HPLC	LTG (12.3), LEV (40.1)
Case 12	Serum	3	LAC	6.5	7.1	HPLC	LTG (8.1), CBZ (9.7), CBZO (3.5)
Case 13	Serum	2	LEV	9.1	8.5	HPLC	PHT (3.2)
Case 14	Serum	2	LEV	9.1	9.1	HPLC	PHT (3.1)
Case 15	Serum	2	LTG	11.9	12.0	HPLC	VPA (80.0)
Case 16	Serum	4	LTG	5.2	4.3	HPLC	LAC (7.6), LEV (30.0), PBT (19.4)
Case 17	Serum	3	PBT	42.3	41.0	GC/MS	PHT (7.1), VPA (13.0)
Case 18	Serum	3	PGR	12.5	13.0	LC–MS-MS	LEV (79.0), ZNS (24.5)
Case 19	Serum	2	PGR	14.6	14.0	LC–MS-MS	GBP (20.1)
Case 20	Serum	3	PGR	2.5	2.4	LC–MS-MS	GBP (0.8), LEV (25.6)
Case 21	Serum	4	RFM	10.3	11.0	LC–MS-MS	ZNS (7.9), LTG (6.7), VPA (67.5)
Case 22	Serum	2	STP	4.0	3.9	HPLC	VPA (107.0)
Case 23	Serum	4	STP	6.4	5.2	HPLC	ZNS (22.0), RFM (6.7), PBT (25.0)
Case 24	Serum	3	TIG	0.05	0.04	LC–MS-MS	GBP (24.6), (11.8), TPR (11.0)
Case 25	Serum	4	TIG	0.06	0.06	LC–MS-MS	PBT (9.4), LEV (47.0), OXC (<0.05), S-LC (13.9)
Case 26	Serum	2	TPR	10.0	9.6	LC–MS-MS	LEV (15.1)
Case 27	Serum	3	VIG	15	15.3	LC–MS-MS	LEV (12.7), ZNS (42.9), VPA (86.9)
Case 28	Serum	3	VIG	7	7.4	LC–MS-MS	PBT (6.9), CBZ (6.0), CBZO (3.1)
Case 29	Serum	2	ZNS	40.6	40	HPLC	LEV (10.0)
Case 30	Serum	1	ZNS	12.8	13	HPLC	–

taken by the patients and observing any changes in the concentrations, which might result from the drugs interaction, pharmacogenetic variations or from other AEDs taken concomitantly by the patients with or without prescription for non-epileptic medications or for abuse purposes, greater clinical care can be given to these patients.

Conclusion

A simple, accurate and cost-effective LC–MS-MS method has been developed for the simultaneous quantification of 18 AEDs and 4 metabolites in postmortem blood, serum and plasma. Although the method run time is 17 min, the 22 AEDs can be analyzed in on step using a very cheap and simple protein precipitation extraction. The method is suitable for routine forensic toxicology and TDM and has been successfully verified using authentic case samples.

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Extraction optimization of 15 antiepileptic drugs and two selected metabolites in postmortem whole blood using LC/MS/MS.

Shaza Deeb, Denise A. McKeown, Hazel J. Torrance, Fiona M. Wylie, Karen S. Scott

Background/Introduction:

In recent years, there has been a growth in reports of antiepileptic drugs (AEDs) being misused in a variety of toxicological case types such as drug abuse and drug facilitated crime. Some of these AEDs are part of routine forensic analysis e.g. carbamazepine however many are not. This study aims at comparing seven different protein precipitation methods for the simultaneous extraction of the most commonly encountered AEDs in post mortem whole blood namely, lacosamide, eslicarbazepine, retigabine, gabapentin, pregabalin, topiramate, tigabine, lamotrigine, zonisamide, valproic acid, levetiracetam, vigabatrin, oxcarbazepine, carbamazepine and its metabolite carbamazepine-10, 11-epoxide, phenytoin and its metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin (p-HPPH).

Objective:

The aim of this project was to optimize the protein precipitation extraction of 15 AEDs to create an efficient routine forensic toxicological method.

Methods:

A 100 µl aliquot of whole blood spiked with a mixture of 17 AEDs was extracted using 7 different solvent mixtures with 2 different centrifuging speeds for 3 different periods. Samples were analysed using a triple quadruple LC/MS/MS and a Gemini Phenomenex C 18 column with 2mM ammonium acetate and methanol as the mobile phase.

Results/Conclusion:

The precipitants found to yield highest recoveries with lowest standard variations were methanol or acetonitrile. Extraction with methanol centrifuged at 5000 rpm for 10 minutes was considered as the method of choice because it is cheaper and simpler (200 µL of the extract is diluted with 1.5 mL water and injected directly into the LC/MS/MS). The method has been used for routine toxicological analysis of postmortem cases.

Determination of 15 antiepileptic drugs and two selected metabolites in postmortem whole blood using LC/MS/MS.

Shaza Deeb, Denise A. McKeown, Hazel J. Torrance, Fiona M. Wylie, Karen S. Scott

Background/Introduction:

In recent years, there has been a growth in reports of antiepileptic drugs (AEDs) being misused on their own or in combination with other drugs of abuse such as heroin and other opioids in a variety of toxicological case types such as drug abuse, suicide, overdose and drug facilitated crime. Some of these AEDs are part of routine forensic analysis e.g. carbamazepine and phenytoin however many are not. A number of laboratory methods have been described to quantify AEDs and their metabolites but all of these are for therapeutic drug monitoring purposes or for one drug and its metabolites. To our knowledge, there are no simultaneous quantification methods for the simultaneous analysis of the most commonly encountered AEDs in post mortem whole blood to include lacosamide, eslicarbazepine and retigabine in addition to gabapentin, pregabalin, topiramate, tigabine, lamotrigine, zonisamide, valproic acid, levetiracetam, vigabatrin, oxcarbazepine, carbamazepine and its metabolite carbamazepine-10, 11-epoxide, phenytoin and its metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin (p-HPPH).

Objective:

The aim of this project was to develop and validate a method for the determination of 15 AEDs and two metabolites in whole blood using liquid chromatography triple quadruple tandem mass spectrometry (LC/MS/MS) which would be suitable for routine forensic toxicological analysis.

Methods:

A 100 µl aliquot of the sample was transferred to a 2-mL snap top polypropylene microcentrifuge tube, 50 µL of internal standards solution (Gabapentin–D10, Tolbutamide and 10, 11 dihydrocarbamazepine) and 250 µL methanol were added, vortexed for 30 seconds and centrifuged for 10 minutes at 5000 rpm. An aliquot of 200 µL of the supernatant was transferred to LC vial and diluted with 1.5 mL of deionized water. A 5 µL of the diluted supernatant was injected into an Agilent LC/MS/MS 6420 triple quadruple system coupled with an Agilent 1200-series LC system. Electrospray ionization (ESI) was

used in dynamic multiple reaction monitoring mode with ion mode switching. The column used was a Gemini Phenomenex –C18 (150 mm x 2.1 mm, 5 μm). Gradient elution was chosen using a mobile phase consisting of 2mM ammonium acetate and methanol at a flow rate of 0.3 mL/min. The total run time was 20 minutes with a column temperature maintained at 40 °C.

Results:

All AEDs were detected and quantified within 20 minutes without endogenous interferences. The linear range for each AED was as follows: valproic acid, vigabatrin and levetiracetam: 5-300.0 mg/L; carbamazepine, carbamazepine-10, 11-epoxide, eslicarbazepine, topiramate, lamotrigine, lacosamide, pregabalin and gabapentin: 0.5-50 mg/L; zonisamide, phenytoin and its metabolite p-HPPH: 1-50 mg/L. Oxcarbazepine and tigabine 0.05-10 mg/L; retigabine: 0.5-10 mg/L. The correlation coefficient (R2) was greater than 0.994 for all AEDs with accuracy and precision exceeding 85% ±15% for all analytes. The recovery ranged from 50% to 98%. No carryover was observed in a blank injected after the highest standard and the matrix effect was acceptable and ranged from 90% to 120%.

Conclusion/Discussion:

A simple, accurate, and sensitive LC-MS/MS method has been developed for the simultaneous quantification of 15 AEDs and 2 metabolites. The method has been used for routine toxicological analysis of postmortem cases.

Key words: Antiepileptic drugs, drug abuse, LC/MS/MS, postmortem blood.

Determination of 22 antiepileptic drugs in postmortem blood, serum and plasma using LC/MS/MS with focus on their rule in forensic cases.

Shaza Deeb; Denise A. McKeown; Hazel J. Torrance; Fiona M. Wylie; Barry K. Logan; Karen S. Scott.

In recent years, antiepileptic drugs (AEDs) have been associated with an increased likelihood of off-label prescription in non-epilepsy disorders even though most of these indications are still under investigation. The widespread use of the new generation of AEDs, particularly in patients with psychiatric disorders, often for unlicensed indications, increases the risk of self-poisoning, suicide and drug abuse. AEDs are a group of legal prescription drugs which are found in abuse cases either alone or with alcohol or other common drugs of abuse in order to enhance their effects. Drugs fitting this description include gabapentin and pregabalin.

Sudden unexplained death in epilepsy (SUDEP) is a major cause of death among epileptic patients. It is responsible for 18% of epileptic–related deaths. The second most important factor after the frequency of seizures is the number of AEDs taken concomitantly. Furthermore, many of the AEDs might impair driving if their concentrations are not maintained properly; therefore this group of drugs should be tested in cases of suspected impaired driving.

This presentation provides an overview of these drugs and details the development and validation of a quantification method for the analysis of the most commonly encountered AEDs in post mortem whole blood, serum and plasma which would be suitable for routine forensic toxicological analysis and therapeutic drug monitoring at the same time.

Gabapentin and Pregabalin Prevalence Among Prisoners in Scotland: An Insight Into Their Abuse Potential

Shaza Deeb*, Fiona M. Wylie, Karen S. Scott

In recent years, there has been a growth in reports of antiepileptic drugs (AEDs) being misused on their own or in combination with other drugs of abuse such as heroin and other opioids in a variety of toxicological case types such as drug abuse, suicide, overdose and drug facilitated crime. Pregabalin and gabapentin abuse cases have significantly increased among drug abusers and prisoners since 2011. Both medicines are indicated for epilepsy, neuropathic pain and generalised anxiety disorder. The latter two indications are the more common in primary care and prison settings.

In order to evaluate the prevalence and abuse potential of AEDs among prison populations in Scotland, a total of 904 urine samples were collected from 8 prisons in Scotland over a one month period (November 2013). These samples were collected initially by the Scottish Prison Service to evaluate the prevalence of other illicit drugs. Prisons that participated were Perth (187 samples), Barlinnie (172 samples), Polmont (151 samples), Low Moss (118 samples), Corton Vale (101 samples), Edinburgh (85 samples), Addiwell (63 samples), and Greenock (27 samples). After the samples have been tested by the SPS, they were stored at -20°C until the analysis time.

Samples were analysed using a simple and accurate method which has been developed and validated for the simultaneous quantification of 22 AEDs (carbamazepine and its metabolite carbamazepine-10,11-epoxide, eslicarbazepine acetate and its metabolite S-licarbazepine, gabapentin, lacosamide, lamotrigine, levetiracetam, oxcarbazepine and its metabolite 10,11-dihydro-10-hydroxy carbamazepine, pregabalin, phenobarbital, phenytoin and its metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin (p-HPPH), retigabine and its metabolite N-acetyl retigabine, rufinamide, stiripentol, topiramate, tigabine, valproic acid, vigabatrin and zonisamide) in urine using LC/MS/MS-QQQ (JAT-14-1592.R1).

Total AEDs prevalence ranged from 4% (Greenock) to 27% (Perth). Gabapentin was identified in 118 samples (13%) and pregabalin in 32 samples (4%). Interestingly, 12 samples contained both drugs (7%). The concentrations ranged from 0.4-1100 mg/L (median: 15 mg/L) for gabapentin and from 0.4-440 mg/L (median: 7.3 mg/L) for pregabalin. Four samples were found to be higher than 400 mg/L. These concentrations are

at least 20 times above the median concentrations and it could at least be suspected that those individual took higher doses than recommended. Other AEDs detected were levetiracetam (4 samples), vigabatrin (4 samples), lamotrigine (3 samples), valproic acid (3 samples), carbamazepine (2 samples) and topiramate (1 sample).

The positive samples were compared with the SPS results in order to see other illicit drugs associated with AEDs. 81 % of these samples have at least one illicit drug. Benzodiazepine, opiate and cannabis were dominating with a percentage of 61, 54 and 47 % respectively. All these drugs were not prescribed. Unprescibed Methadone was positive in 26% of the samples. Cocaine and buprenorphine were positive in 18 and 17% of the samples, whereas amphetamines, methamphetamines and barbiturates only found in 4% of the positive AEDs samples.

In conclusion, this study shows a high prevalence of AEDs (18 %) majorly due to gabapentin and pregabalin on their own or in combination with other drugs of abuse.

Key words: Gabapentin, pregabalin, antiepileptic drugs, drug abuse, LC/MS/MS, urine.

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"Simultaneous Analysis of 22 Anti Epileptic Drugs in Postmortem Blood, Serum and Plasma Using LC/MS/MS with a Focus on their Role in Forensic Cases"

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